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Original Article

Cell-free fat extract protects septic lethality via restraining NLRP3 inflammasome activation

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Abstract: Background: Sepsis is a dysregulated host response to infection with high mortality and current management cannot reach optimal remission. Previous studies have shown that cell-free fat extract (CEFFE) is a kind of bioactive extraction from adipose tissues and exhibits a potent anti-inflammatory effect on wound healing and inflammatory diseases. However, the potential role of CEFFE in sepsis remains unclear. Methods: CEFFE was extracted from healthy donors and was intraperitoneally injected into septic mice. The septic mice models were constructed using lipopolysaccharide (LPS), E. coli, and cecal ligation and puncture (CLP). The survival of septic mice was detected for 96 h and Kaplan-Meier analysis was used to analyze the differences of survival rates. Lung tissues that were collected from septic mice were subjected to HE staining to evaluate the extent of lung injury, and the mice serum was obtained for inflammasome-related cytokines detection. Moreover, peritoneal macrophages were extracted from C57 mice and treated with CEFFE and/or inflammasome activators. The level of IL-1β, IL-18, IL-6, and TNF-α was detected by ELISA, and the activation of NLRP3 were evaluated by Western Blot. Total mtDNA and mitochondrial permeability transition pore were determined to explore the mitochondrial dysfunction in the activation of NLRP3 inflammasome with or without CEFEE. Coimmunoprecipitation (Co-IP) assays were performed to confirm the mechanism of NLRP3 activation induced by CEFFE. Results: CEFFE significantly improved the survival of sepsis mice and alleviate sepsis-induced lung injury. Moreover, CEFFE significantly decreased the level of inflammasome-cytokines (IL-1β and IL-18) but not the pro-inflammatory cytokines such as IL-6 and TNF-α. Moreover, CEFFE markedly suppressed the canonical activation of NLRP3 inflammasome without affecting inflammasomes NLRC4 and AIM2. Additionally, the non-canonical activation of NLRP3 inflammasome was significantly inhibited by CEFFE. CEFFE treatment attenuated the mtDNA outflow and the increase of mitochondrial permeability induced by both canonical and non-canonical pathway of NLRP3 inflammasome activation. The results of Co-IP assays revealed that CEFFE remarkably attenuated the oligomerization of ASC and inhibited the association between NLRP3 and ASC. Conclusion: Our study revealed that CEFFE could significantly alleviate sepsis-related injuries possibly by suppressing NLRP3 inflammasome activation. CEFFE was a promising approach for sepsis treatment.

Keywords: Cell-free fat extract, sepsis, inflammasomes, NLRP3

Introduction

Sepsis is defined as a dysregulated host response to infection and may eventually lead to life-threatening organ dysfunction [1]. Sepsis is a complicated syndrome characterized by physiologic, pathologic and biochemical abnormalities as well as critical illness and high mortality worldwide. The incidence of sepsis continues to remarkably increase, and the costs of sepsis management were over $20 billion in United State each year [2]. Especially for elder patients, they may suffer from long-term physical and cognitive disabilities [3]. Therefore, sepsis is a significant public health concern. Currently, the treatment of sepsis includes antimicrobial therapy, fluid resuscitation, and management of organ dysfunction, but the efficacy

is limited which is hard to reach satisfacory remission. Consequently, there is an urgent need to explore novel, safe and effective therapeutics.

Recently, adipose tissue-derived stromal cells (ADSCs) showed promising efficacy in ameliorating sepsis-induced multiple organ injuries, indicating the potential therapeutic role of adipose tissue in sepsis [4-6]. A fat emulsion, termed nanofat, produced through mechanical forces, has been demonstrated to significantly improve the quality of skin graft in treating skin diseases with an abundant quantity of ADSCs [7]. However, safety concerns and immune rejection remain a challenge regarding cell-based therapies. Herein, *Yu* et al. firstly developed a cell-free fat extract (CEFFE) that was derived from nanofat and exhibited potent therapeutic effects in limb ischemia [8]. Compared with adipose tissues and their derivatives, we characterized the CEFFE with ELISA and proteomic data analyses, and found that CEFFE is cell-free and contains various growth factors, including BDNF, GDNF, TGF-β, HGF and VEGF. Moreover, functional annotation revealed that 56 proteins were involved in angiogenesis. Conventional therapies using viable cells remain a challenge since cell qualities vary from passage to passage. This can be overcome easily by CEFFE by evaluating the content of growth factors. Moreover, the application of CEFFE is risk free for tumorigenesis [8-10]. Therefore, CEFFE is a promising therapeutic agent. Our previous work demonstrated that CEFFE could significantly promote the recovery of diabetic wound and reduce the infiltration of CD68 macrophage, indicating an anti-inflammatory role of CEFFE [11-13]. Since inflammation plays a pivotal role in the development of sepsis, CEFFE is proposed to be a potential therapeutic approach for sepsis. However, the role of CEFFE and its underlying mechanism in sepsis treatment remain unclear.

In this study, we constructed three types of septic mice models, including LPS-induced endotoxemia, E. coli-induced bacteremia, and cecal ligation and puncture (CLP)-induced peritonitis. The application of CEFFE significantly reduced the mortality of septic mice and attenuated sepsis-induced lung injury, which might be mediated by the suppression of canonical and noncanonical activation of NLRP3 inflammasome. The protective role of CEFFE in sepsis via regulation of NLRP3 inflammasome activation, suggested a therapeutic potential of CEFFE for sepsis.

Methods

Preparation of CEFFE

The preparation of CEFFE was mainly conducted as previously described [12]. In brief, adipose tissues extracted from healthy donors were rinsed using saline and centrifuged at 1200 × g for 3 min. The fat located at the middle layer was collected. Then, cells were cracked by a freeze (-80°C)/thaw (37°C) process. After the centrifuge at 2000 × g for 5 min, CEFFE located at the third aqueous layer was collected for further experiments. The protein concentration of CEFFE was determined by BCA kit according to the manufacture manufacturer's protocol from YEASEN. The dose of CEFFE used in experiments were counted by the total protein quantity.

Construction of septic mice model

Three septic mice models were constructed by 8-week-old C57 male mice [14]. LPS (20 mg/kg) was applied to induce endotoxemia; E. coli (1 × 10^8/CFU) was intraperitoneally injected to induce bacteremia; cecal ligation and puncture (CLP) was performed to induce sepsis. For CLP-induced sepsis, mice were anesthetized and the abdomen was incised. Then the cecum was isolated and ligated. Meanwhile, the cecum was punctuated with two holes and its content was brought into the enterocoelia. For the survival assays, 500 μg CEFFE or saline was intraperitoneally injected every 24 h after the septic model construction, and the rest alive mice were sacrificed in 100 h. For ELISA assays, 250 μg or 500 μg CEFFE was intraperitoneally injected one hour before or after the sepsis model construction, and the blood was sampled from mice eyes in the 14 h after the sepsis model construction. This study was approved by the Ethics Committee of Xiangya Hospital, Central South University. All procedures were conducted in accordance with the Institutional Animal Care and Use Committee of Central South University.

Hematoxylin-Eosin (HE) staining

In survival assay, lung tissues were collected and incubated in paraffin after the death of

mice. The paraffin was sliced into 5-mm thick sections and stained with hematoxylin and eosin.

Cell culture

Peritoneal macrophages were extracted from male C57 mice. Before extraction, mice were intraperitoneally injected with 3% thioglycolate. After three days, peritoneal exudate cells were collected and incubated. The adherent monolayer cells were harvested as peritoneal macrophages. HEK293T cells were cultured in DMEM, and THP-1 cells were cultured in RMPI-1640 medium with 10% fetal bovine serum and treated with 100 nM PMA (phorbol-12-myristate-13-acetate) for 3 h to acquired differentiated THP-1 cells.

The activation of NLRP3 inflammasomes

The activation of different inflammasomes in peritoneal macrophages and THP-1 cells was conducted as previously described [15]. Briefly, to activate NLRP3 inflammasome in canonical pathway, 5 * 10^4 cells were incubated with 100 ng/mL LPS for 3 h followed by 5 mM ATP for 1 h, 10 μM Nigericin for 1 h, or 200 μg/mL monosodium urate (MSU) for 6 h. As for non-canonical NLRP3 inflammasome activation, cells were treated with 100 ng/mL Pam3CSK4 for 4 h followed by the application of 2 μg/mL LPS with 5 μg/mL Cholera Toxin Subunit B (CTB + LPS) or LPS transfection using FuGENE HD reagent (Promega) for 16 h. Besides, cells were transfected with 1 μg/mL poly (dA:dT) to activate AIM2 inflammasome or 100 ng/mL LPS followed by 2 μg/mL flagellin to activate NLRC4 inflammasome. Different concentration of CEFFE (100 or 250 μg) was added in the beginning of the induction.

Western blot

Cells were lysed in RIPA buffer (Beyotime, China). The protein was separated in 10% SDS-PAGE gel and transferred to PVDF membranes. The 5% non-fat milk was applied to the membrane to block non-specific antigens. After incubation with primary and secondary antibodies, the protein content was detected using an ECL kit (Pierce, USA). Primary antibodies used in this study were as follows: caspase-1 (ab-179515, Abcam); NLRP3 (Cryo-2, Adipogen); ASC (AL177, Adipogen); IL-1β (AF-401-NA, RD); β-actin (8H10D10, CST).

ELISA

The level of interleukin-1β (IL-1β), IL-18, IL-6, and tumor necrosis factor-α (TNF-α) was detected using ELISA kit (Abcam) according to the manufacturer's instructions.

ASC oligomerization

Peritoneal macrophages were plated in 6-well plates (2 × 10^6 cells per well) and stimulated with 100 ng/mL LPS for 3 h, followed by stimulation with or without 10 μM nigericin for 1 h. Triton Buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 0.1 mM PMSF and EDTA-free protease inhibitor cocktail] was used for the cell lysis at 4°C for 10 min, then 30 μL were removed as whole cell lysates. The rest of cell lysates were centrifuged at 6,000 × g at 4°C for 15 min, the supernatant was disposed, and pellet was washed twice and re-suspended in 200 μL Triton Buffer. Disuccinimidyl suberate (DSS) was used for cross-link for 30 min at 37°C. The pellets were collected and dissolved in SDS loading buffer for Western Blot assays.

Total mtDNA measurement

Total DNA was obtained using Allprep DNA/RNA Mini Kit (Qiagen) according to manufacturer's protocol. Primers specific for the mitochondrial D-loop region or the non-nuclear DNA inserted mtDNA region were used for the mtDNA qPCR determination. Tert and B2m were used for the normalization. The process of qPCR was performed as the previous published study [15]. The primer sequences were as follows: D-loop Forward: 5'-AATCTACCATCCTCCGTGAAACC-3'; D-loop Reverse: 5'-TCAGTTTAGCTACCCCCAAGTTTAA-3'; Tert Forward: 5'-CTAGCT CATGTGTCAAGACCCTCTT-3'; Tert Reverse: 5'-GCCAGCACGTTTCTCTCGTT-3'; B2m Forward: 5'-ATGGGAAGCCGAACATACTG-3'; B2m Reverse: 5'-CAGTCTCAGTGGG GGTGAAT-3'; non-NUMT Forward: 5'-CTAGAAACCCCGAAACCAAA-3', and non-NUMT Reverse: 5'-CCAGCTATCACCAAGCTCGT-3'.

Mitochondrial permeability transition pore (MPTP) detection

Cells (10^5/well) were seeded in 96-well plates, and MPTP opening was investigated following the instructions of Mitochondrial Permeability Transition Pore Assay Kit (Biovision) with some changes [16]. Briefly, calcein-AM solution was diluted to 500 nM with solution buffer. Cells

were washed once with wash buffer and incubated with 50 μL calcein-AM solution for 20 min at 37°C. For washing the excessive calcein-AM, 50 μL CoCl2 solution was added, and cells were incubated 10 min at 37°C. OD at 490 nm of each well was detected, and the MPTP opening level of each group was relative to mock group.

Coimmunoprecipitation (Co-IP)

Total protein of cells was extracted using RIPA lysis buffer supplemented with protease and phosphatase inhibitors and incubated with capture antibody that could recognize the bait protein at 4°C overnight to form an ab-bait-target complex. Magnetic beads (Millipore) were used to separate the complexes from the protein pool. Then, the complexes were eluted from the beads and dissociated by boiling in SDS. To assess the presence or absence of target protein, we performed a Western blot assay. Mouse anti-ASC (Santa Cruz), anti-NEK7 (Santa Cruz) and anti-Flag (abcam) were used as the capture antibodies.

Plasmids and transfection

The NLRP3 full-length sequence was constructed using the following primers: forward, 5'-AACGGGCCCTCTAGACTCGAGATGACGAGTGTCCGTTGCAAGCTGGCTCAGTA-3', reverse, 5'-TAGTCCAGTGTGGTGGAATTCCCAGGAAATCTCGAAGACTATAGTCAGCTCA-3'. The ASC full-length sequence was constructed using the following primers: forward, 5'-AACGGGCCCTCTAGACTCGAGATGGGGCGGGCACGAGATGCCATCCTGGA-3', reverse, 5'-TAGTCCAGTGTGGTGGAATTCGCTCTGCTCCAGGTCCATCACCAAGTAGG-3'. The sequence was amplified and cloned into pcDNA3.1 vectors (GeneChem) with tags. Cell transfection were performed using Lipofectamine™ 3000 according to the manufacturer's protocol.

Statistical analysis

Data were analyzed and visualized by GraphPad Prism 8.0. Data were presented as mean ± SD. Student's t-test was used to compare the differences between two groups, and one-way ANOVA test was used for the comparison of more than two groups. Kaplan Meier analysis was adopted to compare survival differences between two groups. Two-sided *P*-value <0.05 was statistically significant.

Results

CEFFE prevents septic lethality and sepsis-induced lung injury in vivo

To explore the role of CEFFE in sepsis, we applied LPS-induced endotoxemia, E. coli-induced peritonitis and CLP to establish septic model in vivo, and CEFFE or saline were subjected to lethal septic model. We observed that CEFFE treatment significantly prompted the survival rate of septic mice ($P<0.05$). Moreover, CEFFE markedly attenuated endotoxemia-induced lung injury in LPS induced endotoxemia (**Figure 1A**), E. coli-induced peritonitis (**Figure 1B**) and CLP-induced sepsis (**Figure 1C**). Therefore, CEFFE could improve sepsis mobility and protect sepsis-induced organ injury.

CEFFE inhibits the release of inflammasome-related cytokines in sepsis in vivo

Multiple studies found that over activated inflammasome was an important characteristic of sepsis [17, 18]. A previous study showed that NLRP3 inflammasome-related cytokines were upregulated in sepsis, and the reduced level of cytokines promoted the sepsis survival rate and prevent endotoxemia-induced lung injury [19]. Therefore, we further identified whether CEFFE affected cytokines levels in sepsis. CEFFE was applied to mice 1 h before or after the septic model construction with two different dosages (250 or 500 μg). Our results showed that CEFFE significantly reduced the release of IL-1β and IL-18 no matter CEFFE dose and treatment time ($P<0.05$), whereas the release of TNF-α and IL-6 was not affected in LPS induced endotoxemia (**Figure 2A**), E. coli-induced peritonitis (**Figure 2B**) and CLP-induced sepsis (**Figure 2C**). Upon stimulation, activated NLRP3 inflammasome induced IL-1β and IL-18 cleavage and release, and we subsequently wonder whether CEFFE affect NLRP3 activation in vitro.

CEFFE specifically reduces canonical activation of NLRP3 inflammasome

Due to the vital role of NLRP3 inflammasome in sepsis and the previous results, we further identified whether the molecular mechanism of CEFFE protecting sepsis lethality and decreasing cytokines release was related to NLRP3 inflammasome. Under canonical NLRP3 inflammasome stimulus (ATP, nigericin, or MSU), the

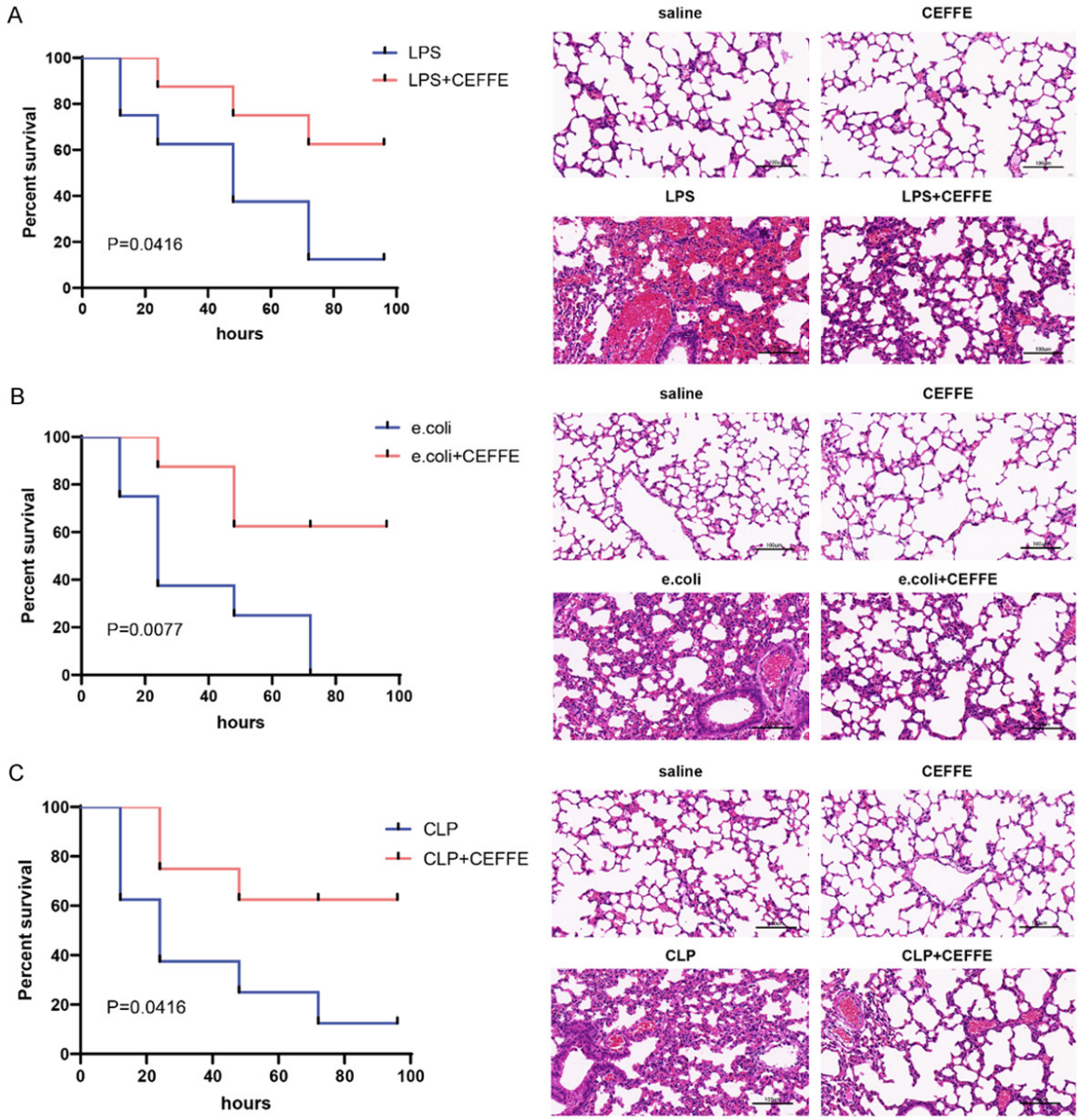

Figure 1. CEFFE significantly improved the survival of sepsis mice and reduced lung injury. The survival time and lung tissues HE staining of sepsis model mice treated with CEFFE (500 μg) or saline. The sepsis models were constructed by LPS (A), E. coli (B), and CLP (C). LPS, Lipopolysaccharide; CLP, Cecal ligation and puncture. *P<0.05.

application of CEFFE with two different dosages (100 or 250 μg) on primary peritoneal macrophage significantly reduced the release of IL-1β and IL-18 (P<0.05) (**Figure 3A**, **3B**) but did not change the level of TNF-α and IL-6 (**Figure 3C**, **3D**). Western blot revealed that CEFFE could significantly reduce the cleavage of IL-1β and caspase-1, whose release was activated by canonical activation of NLRP3 inflammasome (**Figure 3E**). Mitochondrial dysfunction is the driver of NLRP3 inflammasome activation [20]. Therefore, we investigated whether CEFFE can retrieve the mitochondrial permeability increasing in the canonical activation of NLRP3 inflammasome. Our results showed that CEFFE remarkably attenuate the canonical NLRP3 inflammasome stimulus induced mitochondrial permeability increasing and the mtDNA release (P<0.05) (**Figure 3F**, **3G**). However, there was no significant difference between the two CEFFE concentrations. Moreover, the CEFFE treatment had no effect on either poly (dA:dT)-induced AIM2 inflammasome or flagellin (FLA) transfection-induced NLRC4 inflammasome

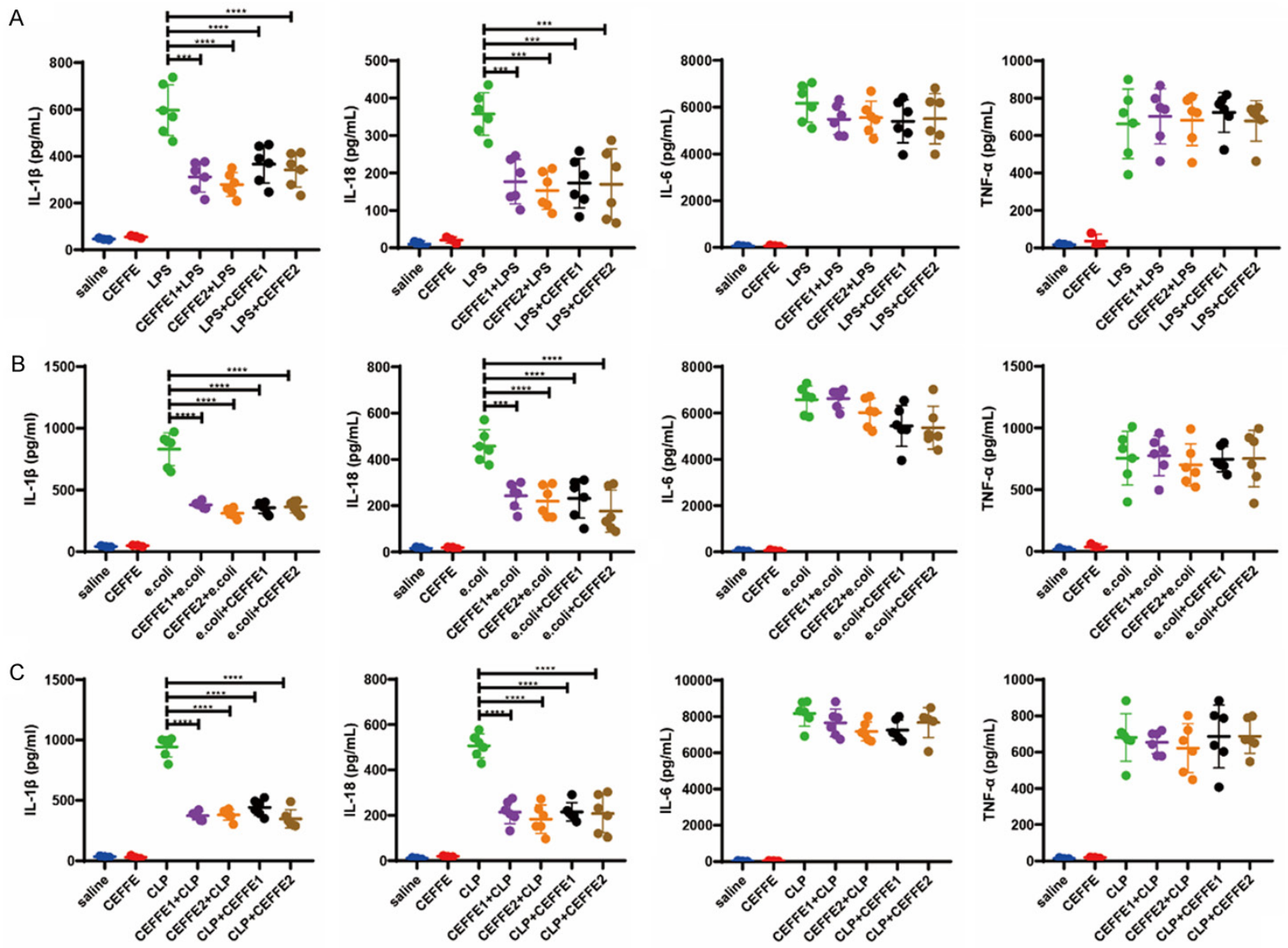
A
IL-1β (pg/mL)
IL-18 (pg/mL)
IL-6 (pg/mL)
TNF-α (pg/mL)
saline
CEFFE
LPS
CEFFE1+LPS
CEFFE2+LPS
LPS+CEFFE1
LPS+CEFFE2
B
IL-1β (pg/ml)
IL-18 (pg/mL)
IL-6 (pg/mL)
TNF-α (pg/mL)
saline
CEFFE
e.coli
CEFFE1+e.coli
CEFFE2+e.coli
e.coli+CEFFE1
e.coli+CEFFE2
C
IL-1β (pg/ml)
IL-18 (pg/mL)
IL-6 (pg/mL)
TNF-α (pg/mL)
saline
CEFFE
CLP
CEFFE1+CLP
CEFFE2+CLP
CLP+CEFFE1
CLP+CEFFE2

Figure 2. CEFFE inhibited the release of inflammasome-related cytokines in sepsis in vivo. The level of IL-1β, IL-18, IL-6, and TNF-α in mice serum treated with CEFFE1 (250 μg) or CEFFE2 (500 μg) 1 h before or after the sepsis model construction. The sepsis models were constructed by LPS (A), E. coli (B), and CLP (C). LPS, Lipopolysaccharide; CLP, Cecal ligation and puncture. ***P<0.001; ****P<0.0001.

activation (**Figure 3H-K**). Meanwhile, the biological activities of CEFFE were investigated in THP-1 cells. The results showed that CEFFE significantly suppressed the ATP, nigericin, or MSU-induced activation of NLRP3 inflammasome (P<0.05) (**Figure 4A-D**) and the stimulus-induced mitochondrial permeability increasing (P<0.05) (**Figure 4E**, **4F**).

CEFFE suppresses non-canonical activation of NLRP3 inflammasome

Except for canonical pathway, upon non-canonical inflammasome activation, NLRP3 is pivotal for IL-1β and IL-18 release dependent on caspase-1 [21]. As expected, different doses of CEFFE (100 or 250 μg) significantly suppressed IL-1β and IL-18 release in macrophages transfected with LPS (P<0.05) (**Figure 5A**, **5B**) without affecting TNF-α and IL-6 level (**Figure 5C**, **5D**). Western blot revealed that CEFFE successfully reduced LPS-induced cleavage of IL-1β and caspase-1, suggesting the suppression of NLRP3 inflammasome activation (**Figure 5E**). Subsequently, mitochondrial permeability transition was determined. Our results showed that CEFFE retrieved mitochondrial permeability increasing and the mtDNA release induced by non-canonical activation of NLRP3 inflammasome (P<0.05) (**Figure 5F**, **5G**). Therefore, our data demonstrate that addition CEFFE treatment specially impairs the non-canonical NLRP3 inflammasome activation.

CEFFE inhibited the activation of NLRP3 inflammasome by impairing ASC oligomerization and association between NLRP3 and ASC

Furthermore, we explored the mechanism of NLRP3 inflammasome deactivation induced by CEFFE. Assembly of NLRP3 inflammasome is a key step during its activation, as demonstrated by ASC oligomerization. Our results showed that CEFFE significantly blocked the oligomerization of ASC in canonical NLRP3 activation of mouse peritoneal macrophages (**Figure 6A**). Upon stimulated, NLRP3 associated with ASC to activate NLRP3 inflammasome. Besides, NEK7 was newly found to combined with NLRP3 to participate the activation of NLRP3 inflammasome. Our results showed that CEFFE reduced the association between NLRP3 and ASC, but not NEK7 (**Figure 6B-D**). Therefore, our results revealed that CEFFE impaired the NLRP3 inflammasome activation by reducing the NLRP3-ASC assembly and ASC oligomerization.

Discussion

Since sepsis is a life-threatening disease and current management is limited, it is necessary to develop novel therapeutics. Our study revealed that CEFFE could significantly improve the survival of septic mice and reduce sepsis-induced lung injury. Meanwhile, CEFFE significantly decreased the level of inflammasome-cytokines (IL-1β and IL-18) while no influence on the level of the pro-inflammatory cytokines such as IL-6 and TNF-α in sepsis mice models. In daddition, CEFFE markedly suppressed the canonical and non-canonical activation of NLRP3 inflammasome by reducing the ASC oligomerization and NLRP3-ASC assembly. Therefore, CEFFE could ameliorate septic lethality via restraining NLRP3 inflammasome activation, which suggested an alternative strategy for sepsis treatment.

Adipose tissue and its derivative, ADSC, were shown to play protective roles in sepsis-induced multiple organ injuries, which indicated a therapeutic potential of adipose tissue derivative in sepsis treatment [4]. As a novel derivative of adipose tissue, CEFFE is cell-free and can avoid multiple concerns in the clinical application of cell-based therapy such as immune rejection, tumorigenesis, and cell viability [8]. Our previous work have illuminated the effective composition of CEFFE and demonstrated the effects of CEFFE on improving the survival of fat graft and ameliorating limb ischemia [8, 9, 11]. The local injection of CEFFE significantly promoted angiogenesis and inhibited the infiltration of CD68 macrophages, which indicated the pro-angiogenic and anti-inflammatory effects of CEFFE [12]. Besides, CEFFE could promote the transition of macrophages from M1 to M2 subtype, which facilitated tissue repair and suppress inflammatory responses [12, 22]. Since

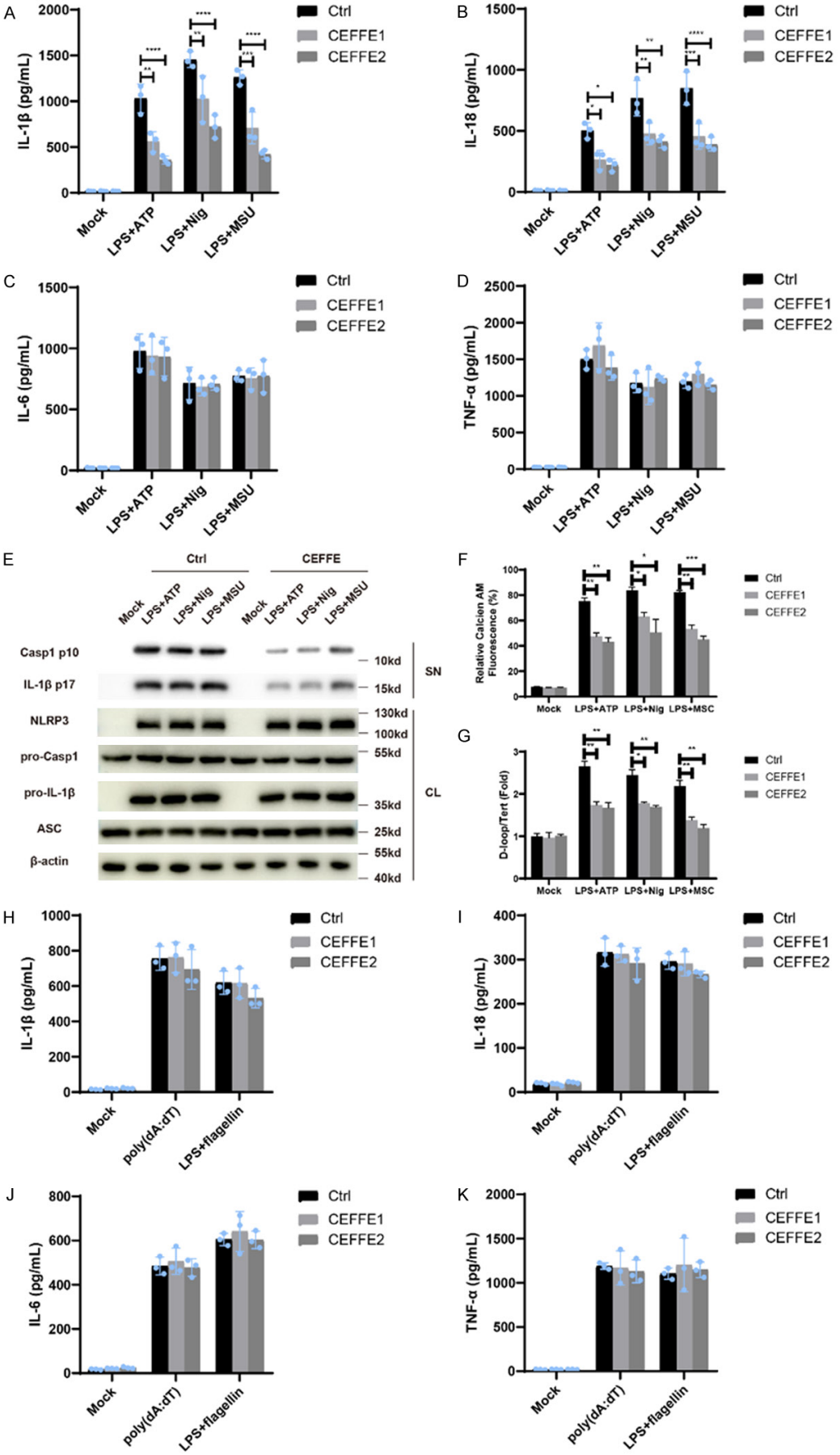
A
IL-1β (pg/mL)
Ctrl
CEFFE1
CEFFE2
Mock
LPS+ATP
LPS+Nig
LPS+MSU
B
IL-18 (pg/mL)
C
IL-6 (pg/mL)
D
TNF-α (pg/mL)
E
Ctrl
CEFFE
Casp1 p10
IL-1β p17
NLRP3
pro-Casp1
pro-IL-1β
ASC
β-actin
10kd
15kd
130kd
100kd
55kd
35kd
25kd
55kd
40kd
SN
CL
F
Relative Calcien AM Fluorescence (%)
LPS+MSC
G
D-loop/Tert (Fold)
H
poly(dA:dT)
LPS+flagellin
I
J
K

Figure 3. CEFFE specifically reduced canonical activation of NLRP3 inflammasome. (A-D) Canonical activation of NLRP3 inflammasome was stimulated using LPS combined with ATP, Nig, or MSU. The level of IL-1β (A), IL-18 (B), IL-6 (C), and TNF-α (D) in supernatants from mouse peritoneal macrophages was detected by ELISA after the application of CEFFE1 (100 μg) or CEFFE2 (250 μg). (E) After canonical activation of NLRP3 inflammasome as previously described, western blot detected the expression of cleaved caspase-1, IL-1β, NLRP3, pro-caspase-1, pro-IL-1β, and ASC in supernatants (SN) or cell lysates (CL) of mice peritoneal macrophages treated CEFFE (250 μg). (F, G) CEFFE retrieved the canonical NLRP3 inflammasome stimulus induced mitochondrial permeability increasing (F) and the mtDNA release (G). (H-K) The effect of CEFFE1 (100 μg) or CEFFE2 (250 μg) on the level of IL-1β (H), IL-18 (I), IL-6 (J), and TNF-α (K) in peritoneal macrophages when poly (dA:dT) was applied to activate AIM2 inflammasome and LPS + flagellin was used to activate NLRC4 inflammasome. *P<0.05; **P<0.01; *** P<0.001; ****P<0.0001.

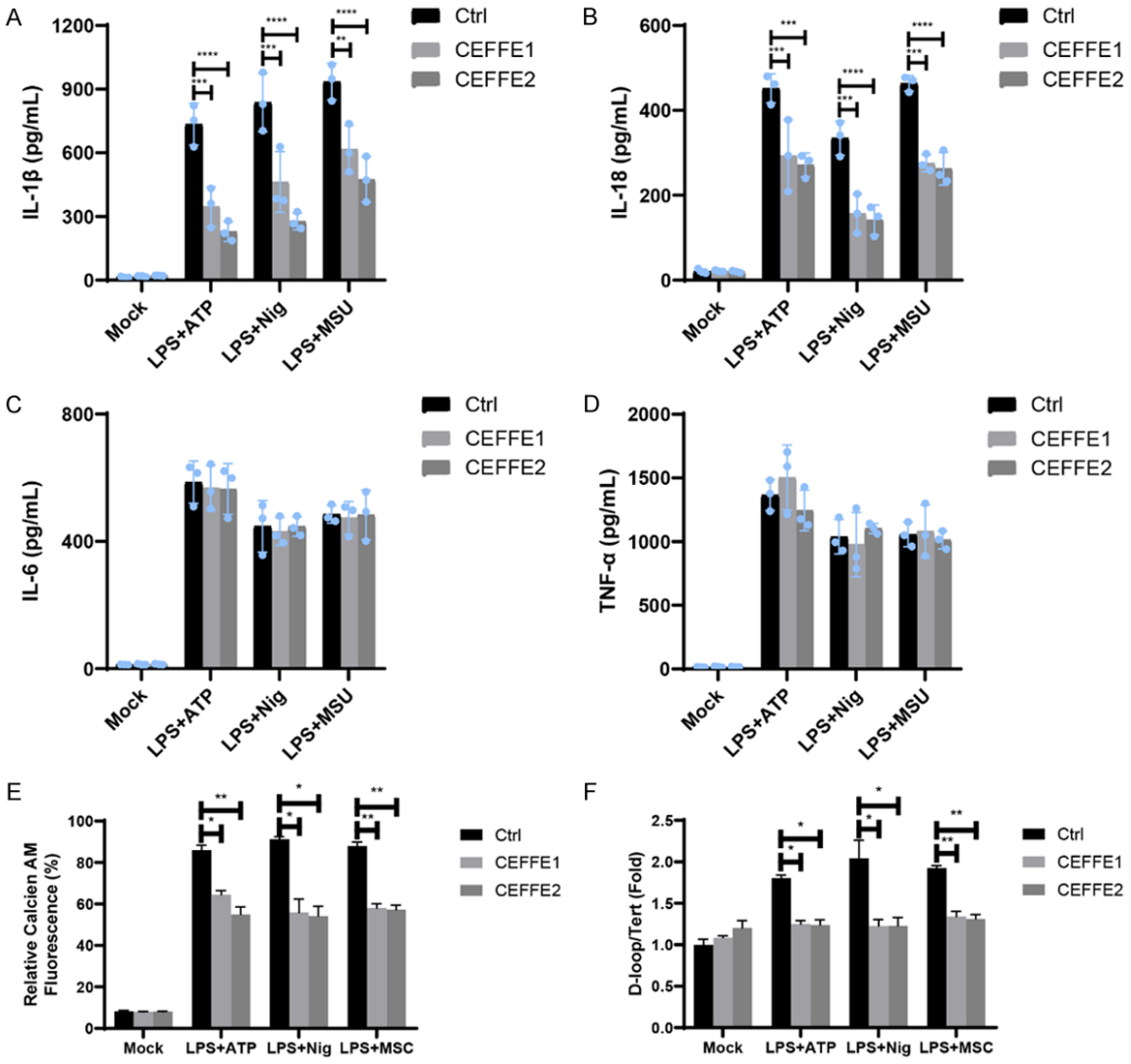

Figure 4. CEFFE inhibited canonical activation of NLRP3 inflammasome in THP-1 cells. (A-D) The effect of CEFFE1 (100 μg) or CEFFE2 (250 μg) on the level of IL-1β (A), IL-18 (B), IL-6 (C), and TNF-α (D) in THP-1 cells treated with LPS and canonical NLRP3 inflammasome stimuli (ATP, Nig, and MSU). (E, F) The effect of CEFFE1 (100 μg) or CEFFE2 (250 μg) on the level of mitochondrial permeability increasing (E) and the mtDNA release (F) induced by canonical NLRP3 inflammasome stimuli. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

sepsis is characterized by overactivated inflammatory responses to pathogens and CEFFE can exert potent anti-inflammatory activities, CEFFE appears to be a potential ideal approach for sepsis treatment [23]. In this study, we established sepsis mice models via LPS-induced

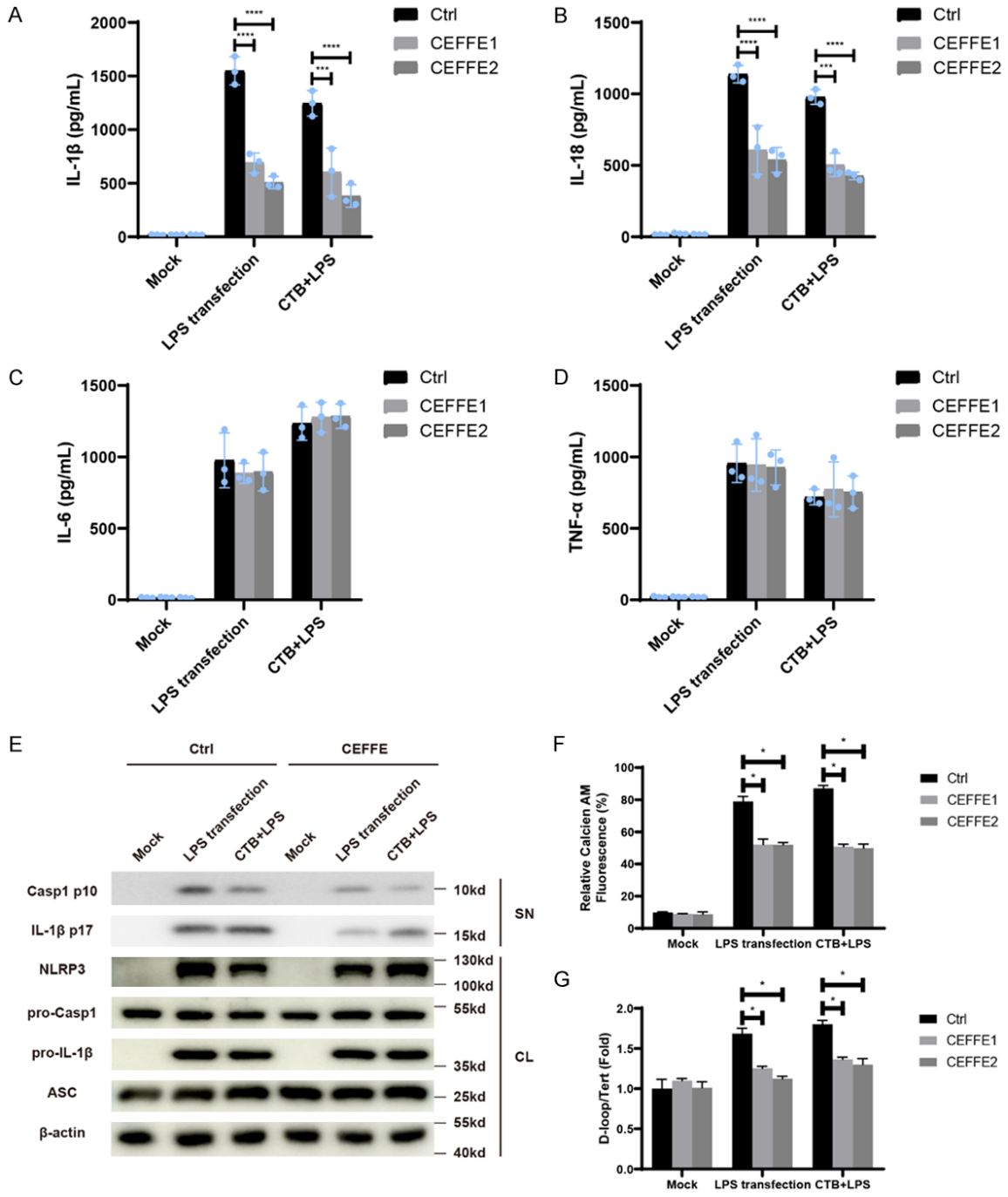

Figure 5. CEFFE suppressed non-canonical activation of NLRP3 inflammasome. (A-D) The non-canonical activation of NLRP3 inflammasome was conducted by transfecting LPS using FuGENE reagent or CTB. The level of IL-1β (A), IL-18 (B), IL-6 (C), and TNF-α (D) in supernatants from mice peritoneal macrophages was detected by ELISA after the application of CEFFE1 (100 μg) or CEFFE2 (250 μg). (E) After LPS transfection, western blot was conducted to detect the expression of cleaved caspase-1, IL-1β, NLRP3, pro-caspase-1, pro-IL-1β, and ASC in supernatants (SN) or cell lysates (CL) of mice-derived macrophages treated with CEFFE (50 μl). (F, G) The mitochondrial permeability increasing (F) and the mtDNA release (G) levels were determined after the application of CEFFE1 (100 μg) or CEFFE2 (250 μg). *P<0.05; ***P<0.001; ****P<0.0001.

endotoxemia, E. coli-induced bacteremia, and CLP-induced sepsis, which efficiently reflected the occurrence of sepsis induced by different stimuli. The application of CEFFE significantly

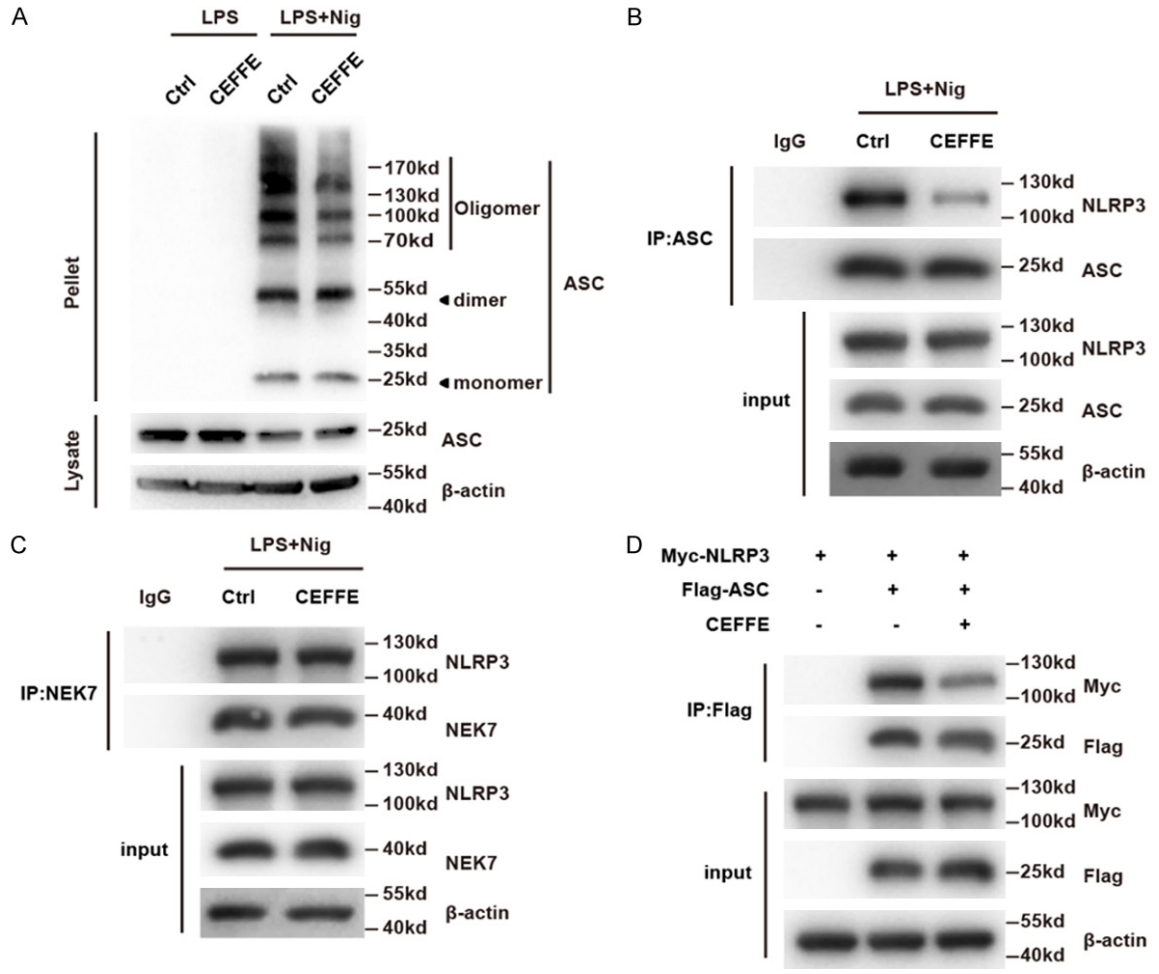

Figure 6. CEFFE inhibited NLRP3 activation by impairing ASC oligomerization and association between NLRP3 and ASC. A. The oligomerization of ASC was significantly impaired by CEFFE stimulation. B. The association between NLPR3 and ASC induced by LPS + Nig was inhibited by CEFFE stimulation in mouse peritoneal macrophages. C. The combination of NLRP3 and NEK7 induced by LPS + Nig was not affected by CEFFE stimulation. D. The association between tagged NLPR3 and ASC was inhibited by CEFFE stimulation in HEK293T cells.

prolonged the survival of sepsis mice and attenuated sepsis-induced lung injury, which was a common type of organ dysfunction [24]. To date, sepsis has no specific therapy but merely respiratory or volume treatments. CEFFE has therapeutic effect in sepsis and can be used for treatment easily because of its autologous extraction and convenience to store. The components CEFFE have no immunogenicity. Therefore, it may be used for patients who have complex immune conditions. Taken together, CEFFE has great prospect of treatment application in sepsis.

Inflammasomes are multimeric proteins that orchestrate inflammation in response to stimuli [25, 26]. A previous study indicated that the expression of NLRP3 inflammasome was significantly elevated in the macrophages of sepsis patients [19]. The inhibition of NLRP3 inflammasome was shown to improve the survival of septic mice and attenuate sepsis-induced organ injury [27, 28]. Moreover, our previous work revealed that the suppression of NLRP3 inflammasome significantly attenuated LPS-induced systemic inflammation and MSU-induced peritonitis [15]. Therefore, NLRP3 inflammasome played a pivotal role in sepsis. In this study, we found that the application of CEFFE significantly reduced the level of inflammasome-related cytokines without affecting other pro-inflammatory cytokines, and the anti-inflammatory effects of CEFFE were independent of LPS and dose. Therefore, CEFFE could exert

protective effects before or after the occurrence of sepsis. When NLRP3 inflammasome was activated in the canonical or non-canonical pathway, the level of IL-1β and IL-18 would be promoted, and this effect could be reversed by CEFFE. Given the fact that the activation of AIM2 and NLRC4 inflammasomes could also lead to the elevation of IL-1β and IL-18, poly (dA:dT) and flagellin were applied to activate AIM2 and NLRC4 inflammasomes. In contrast to NLRP3 inflammasome, the application of CEFFE failed to decrease the secretion of IL-1β and IL-18 with the presence of activated AIM2 and NLRC4 inflammasomes.

In the process of signal 2 NLRP3 inflammasome activation, the mitochondrial dysfunction is a critical biological behavior [29]. The mitochondrial DNA redistribution in cytoplasm induced by permeability transition can activate NLRP3 inflammasome [30, 31]. Our study confirmed that CEFFE treatment can attenuate the increase of mitochondrial permeability induced by both canonical and non-canonical pathway, but not dose-dependently. Moreover, the oligomerization of ASC is a vital mechanical process after the start of NLRP3 inflammasome activation. Our co-IP assays revealed that CEFFE impaired NLRP3 activation by reducing the ASC oligomerization and NLRP3-ASC assembly, but the interaction between NLRP3 and NEK7 was not affected by the CEFFE treatment. Meanwhile, the results of our study were limited by the complicated composition of CEFFE. The specific active components that activated the NLRP3 inflammasome in CEFFE need to be explored in the further research. Therefore, our study demonstrated that CEFFE exerted anti-inflammatory effects by suppressing NLRP3 inflammasome activation via NLRP3-ASC assembly inhibition.

Conclusion

Our study revealed that CEFFE could significantly ameliorate sepsis-induced injuries by suppressing NLRP3 inflammasome activation possibly via NLRP3-ASC assembly inhibition. CEFFE is a potential ideal approach for sepsis treatment.

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Disclosure of conflict of interest

None.

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