MPC1 regulates endotoxin tolerance of macrophages through mitochondrial oxidative stress resistance

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Abstract

Increasing evidence highlights the crucial role of endotoxin tolerance in the regulation of the sepsis, yet its underlying mechanisms remain poorly understood. Here, we provide evidence to support a novel role for the mitochondrial pyruvate carrier1 (MPC1)-mediated mitochondrial oxidative stress resistance in regulation of endotoxin tolerance in macrophages. We observed a decrease in MPC1 expression in both *in vitro* and *in vivo* sepsis models, while LPS-tolerant macrophages exhibited increased MPC1 levels. Overexpression of MPC1 significantly reduced LPS-induced inflammatory responses and oxidative stress, suggesting its anti-inflammatory properties. Intriguingly, we found that overexpression of MPC1 did not foster endotoxin tolerance in macrophages. Furthermore, we found that overexpression of MPC1 inhibited mitochondrial oxidative stress resistance mediated by mitochondrial reactive oxygen species (mtROS), which weakened the resistance of macrophages to LPS secondary stimulation, and then inhibited endotoxin tolerance. Despite mtROS typically acting as pro-inflammatory mediators, our findings indicate that mtROS production, regulated via the SIRT3/SOD2 pathway, is essential for MPC1's control over mitochondrial oxidative stress resistance and endotoxin tolerance. Collectively, these findings uncover novel mechanisms through which MPC1 modulates inflammation and induces endotoxin tolerance, underscoring the potential of targeting MPC1 in sepsis treatment.

Introduction

Sepsis is a systemic inflammatory response syndrome mediated by immune cells, characterized by multiple organ failure, host innate immune dysregulation, and inflammatory responses to infection[1]. An expanded inflammatory response mediated by high concentrations of Lipopolysaccharide (LPS) in the early stages of sepsis, coupled with immune "paralysis" in both the immune system and immune cells in the later stages, are principal causes of mortality in sepsis patients[2, 3]. Endotoxin tolerance is a phenomenon in which the body or immune cells exhibit a diminished or absent response to high or lethal doses of LPS after initial stimulation by a low dose of LPS[4]. This tolerance in macrophages serves as a crucial protective mechanism to limit excessive inflammatory responses in sepsis[5]. However, macrophage endotoxin tolerance is also a "double-edged sword" in the progression of sepsis: on the one hand, endotoxin tolerance provides a protective mechanism to reduce inflammatory damage of tissues in the early stage of sepsis; On the other hand, individuals with persistent endotoxin tolerance is associated with increased mortality rates in the late stages of sepsis[6, 7] The mechanisms underlying these effects require further investigation.

The inflammatory response and endotoxin tolerance in macrophages are intimately linked to mitochondrial function. For example, inhibiting Drp1/Fis1-dependent mitochondrial fragmentation has been shown to enhance macrophage function and immune response in both *in vitro* and *in vivo* models of endotoxin tolerance. This benefit is mediated, at least in part, by decreasing the release of damaged extracellular mitochondria, which contributes to endotoxin cross-tolerance[8]. Our previous studies have demonstrated that the formation of the PKM2 tetramer, induced by TEPP-46—a small molecular activator—promotes endotoxin tolerance in macrophages by enhancing mitochondrial biogenesis[9].

Mitochondrial biogenesis is a hallmark of mitohormesis[10]. Mitohormesis is a process wherein mitochondria adapt to continuous stress following mild stress stimulation, providing both mitochondrial and cellular protection[11]. Mitohormesis plays an important role in regulating many physiological and pathological processes, such as, aging and cancer[12, 13]. In addition, mitohormesis is also involved in the regulation of macrophage immune tolerance[14]. However, it remains unclear whether mitochondrial oxidative stress resistance, another aspect of mitohormesis, plays a role in managing sepsis and endotoxin tolerance. Mitochondrial oxidative stress resistance refers to the adaptation of mitochondria to re-oxidative stress stimulation after mild dose of oxidative stress stimulation, which is an important endogenous protective way to limit the over-activation of mitochondria by oxidative stress stimulation. It is reflected in the production of less mitochondrial reactive oxygen species (mtROS) and reactive oxygen species (ROS) when stimulated by oxidative stress again[14]. ROS is a pro-inflammatory mediator, and ROS is mainly transported to the cytoplasm by mtROS through mitochondrial permeability transition pore (mPTP), so mtROS is also considered as a pro-inflammatory mediator[15]. However, the latest research shows that low concentration of mtROS also has anti-inflammatory and antioxidant properties[14]. These lower concentrations of mtROS may inhibit the mitochondrial oxidative stress response to exogenous oxidants, thereby reducing inflammatory responses[14].

Mitochondrial pyruvate carrier (MPC) is a protein located on mitochondrial membrane, and its main function is to transport pyruvate located in cytoplasm to mitochondria for metabolism[16]. MPC consists of two subunits, MPC1 and MPC2[17]. MPC1 is often abnormally expressed in various cancer tissues, which promotes the growth and metastasis of tumor cells by increasing the transcription of downstream oncogenes[18, 19]. The loss of MPC1 in early thymocytes participated in the development of T cells. Specifically, knocking out of MPC1 in CD4 + T cells can promote the expression of inflammatory factors IFN-γ and IL-17A, and induce autoimmune encephalomyelitis in mice[20]. These findings suggest that MPC1 is an important regulator of inflammation and immune responses. However, the relationship between MPC1 and endotoxin tolerance, and its role in regulating mitochondrial oxidative stress resistance, remain to be elucidated.

This study primarily investigates the role and molecular mechanisms of MPC1 in regulating sepsis and endotoxin tolerance in macrophages. We have found that MPC1 is a critical regulator of both sepsis and macrophage immune tolerance. The expression of MPC1 was observed to decrease in macrophages in both in vivo and *in vitro* models of sepsis. Interestingly, overexpression of MPC1 inhibits the development of sepsis but does not induce endotoxin tolerance in macrophages. This is mainly because the overexpression of MPC1 inhibits the production of mtROS through the SIRT3/SOD2 axis, thereby reducing mitochondrial oxidative stress resistance and ultimately inhibiting endotoxin tolerance in macrophages. These results not only unveil a novel regulatory mechanism of endotoxin tolerance but also suggest a new therapeutic target to combat sepsis-mediated immunosuppression.

Materials and methods

Reagents

For our experiments, we utilized a variety of antibodies including MPC1 (Abcam, ab74871, 1:1,000 and Cell Signaling, #14462, 1:1,000), SIRT3 (Cell Signaling, #5490, 1:1,000), Ac-SOD2 (Abcam, ab137037, 1:2,000), β-actin (Boster, M01263-6, 1:2,000). Secondary antibodies such as Anti-mouse IgG (Abbkine, A23010, 1:5,000) and Anti-rabbit IgG (Abbkine, A21020, 1:10,000) were also employed. We used 1640 medium (Hyclone, SH30809.01B), PBS (Boster, AR0192) and FBS (PAN, P30-3302). Additional materials included Protein Marker (Thermo Fisher, 26616), penicillin–streptomycin (Beyotime, C0223), BeyoECL Plus (Beyotime, P0018S), Cell Counting Kit-8 (Beyotime, C0037), 4-OHE1 (MedChemExpress, HY-W009300), MitoPY1 (Tocris), CellROX® Deep Red (Thermo Fisher, C10422), LPS (Sigma, L9641), 3-TYP (MedChemExpress, HY-108331), mito-TEMPO (MedChemExpress, HY-112879) and Menadione (MedChemExpress, HY-B0332). TNF-a (EK0527) and IL-6 (EK0411) ELISA kits from Boster Biological Technology were used for cytokine quantification. Primers for RT-qPCR were obtained from Sangon Biotech.

Cell Isolation and Culture

Male C57BL/6 mice, 8 weeks old, were procured from the Animal Experimental Center of Chongqing Medical University. Mouse peritoneal macrophages (PMs), Kupffer cells (KCs) and bone marrow-derived macrophages (BMDMs) were isolated from C57BL/6 mice as previously described[21]. The RAW264.7 macrophage cell line was purchased from Boster Biological Technology. These cell populations were cultured in 1640/DMEM medium with 10% FBS and 1% penicillin–streptomycin at 37°C, under 95% humidity and 5% CO2.

Sepsis And Endotoxin Tolerance Models

RAW264.7 cells were pretreated with 100 ng/ml LPS for 6 h, and then treated with 500 ng/ml LPS to establish an endotoxin tolerance model. A sepsis model was similarly developed by treating cells directly with 500 ng/ml LPS. The detection of TNF-α and IL-6 levels in the supernatant occurred 1 hour post-treatment, whereas MPC1, mtROS, and ROS levels were assessed after 3 hours. In vivo models involved an initial intraperitoneal injection of 400 μg/kg LPS for 6 hours, followed by a 4 mg/kg LPS dose to establish endotoxin tolerance in mice. A sepsis model was induced via a single intraperitoneal injection of 4 mg/kg LPS. These studies were approved by the Chongqing University Three Gorges Hospital Institutional Ethics Committee and conducted according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care.

Lentivirus Transduction

The lentivirus overexpressing of MPC1 (OE-*MPC1*), lentivirus overexpressing of SIRT3 (OE-*SIRT3*) and corresponding empty lentivirus were prepared by Sangon Biotechnology (Shanghai). The lentiviral transduction were performed according to the manufacturer's instructions. In brief, 1×10^5 RAW264.7 cells (MOI = 20) in 1 ml medium with 10 mg/ml of polybrene (GeneChem) were incubated with 3 μl lentivirus. After 24 h, the medium was replaced and cells were cultured for an additional 48 hours. The efficacy of transduction was assessed via Western blot and RT-qPCR.

Measurements Of Cell Viability

Cell viability was assessed using the Cell Counting Kit-8 (CCK8). In brief, 5×10^3 of RAW264.7 cells were seeded in each well of a 96-well plate with 100 μl of medium and cultured for 24 h. Then, 10 μl of CCK8 was added to each well, and the cells were incubated at 37°C for 1 h. The absorbance of each well was detected at 450 nm wavelength by using a microplate reader.

Western Blot Assay

Western blot analysis was carried out as previously described[22]. In brief, proteins were extracted from cells with protein extraction kit (KeyGene), and the protein concentration was detected with a BCA protein assay kit (Beyotime) in each group. The protein samples were mixed with 5× loading buffer at a 4:1 volume ratio and boiled for 10 min. Between 10–20 μg of protein per sample was separated by SDS-PAGE on 10% gels and transferred to PVDF membranes (Millipore) at 250 mA. Membranes were blocked with 5% BSA for 20 min at room temperature, then incubated with primary antibodies overnight at 4°C. Following three washes with TBST, HRP-conjugated secondary antibodies were applied for 1 hour at room temperature. Protein bands were visualized by ECL reagent (Beyotime) at room temperature for 5 ~ 30 s and analyzed with Quantity One software (Bio-Rad).

RT-qPCR Assay

The total RNA was extracted from RAW264.7 cells using TRIzol® reagent (ThermoFisher, 15596026). Detect RNA concentration, and then the RNA was reverse-transcribed to cDNA with the PrimeScript™ RT Reagent Kit (TaKaRa Biotechnology, RR047A). Primers for RT-qPCR were obtained from Sangon Biotechnology (Shanghai). The expression of the target gene was normalized to that of GAPDH. The primers used were as follows: MPC1, forward 5’-GACTTTCGCCCTCTGTTG-3’ and reverse 5’-GTCGTCCTCCCTGAATGA-3’; TNF-α, forward 5’-CCCTCACACTCAGATCATCTTCT-3’ and reverse 5’-GCTACGACGTGGGCTACAG-3’; IL-6, forward 5’-TAGTCCTTCCTACCCCAATTTCC-3’ and reverse 5’-TTGGTCCTTAGCCACTCCTTC-3’; and GAPDH, forward 5’-AGGTCGGTGTGAACGGATTTG-3’ and reverse 5’-TGTAGACCATGTAGTTGAGGTCA-3’. The relative gene expression was analyzed using the $2^{-\Delta\Delta Cq}$ method.

Flow Detection Of mtROS And ROS

MtROS levels were assessed using the MitoPY1 probe. RAW264.7 cells were treated with 10 μM MitoPY1 for 1 h, then placed on ice, and stained with DAPI to facilitate mtROS detection by flow cytometry. Following MitoPY1 incubation, 500 μM H2O2 was added for 10 min as a positive control. ROS levels were detected using the CellROX probe; cells were treated with 1.25 μM CellROX for 30 min, followed by the addition of DAPI for flow cytometric analysis. As a positive control for ROS, 500 μM H2O2 was added 20 min after CellROX treatment.

Cytokine Measurements.

Concentrations of TNF-α and IL-6 in serum or culture supernatant were quantified using ELISA kits (Boster, EK0527 for TNF-α and EK0411 for IL-6) according to the manufacturer’s instructions.

Cell Immunofluorescence Experiment

RAW264.7 cells were treated with 0, 10, or 50 μM menadione for 1 h at room temperature, followed by incubation with 5 μM CellROX® Deep Red probe for 30 min. Cells were then washed with PBS and visualized using a fluorescence microscope.

Statistical Analysis

Statistical analyses were performed using SPSS 17.0 software (SPSS, Inc.). Data were analyzed using Student's *t*-test or ANOVA where appropriate. All data, unless specified otherwise, are presented as mean ± SEM. A statistically significant difference was defined as $p < 0.05$.

Results

MPC1 was inhibited in sepsis and endotoxin tolerance models

In our previous study, we discovered that the formation of the pyruvate kinase M2 (PKM2) tetramer plays a crucial role in inducing endotoxin tolerance in macrophages[9]. PKM2 primarily functions to catalyze the conversion of phosphoenolpyruvate into pyruvate, which is then transported into mitochondria by mitochondrial pyruvate carrier 1 (MPC1) to fuel the tricarboxylic acid cycle (TAC)[23]. Research has shown that MPC1 is intimately associated with the development of acute inflammation[14]. However, whether MPC1 is involved in the regulation of sepsis and endotoxin tolerance of macrophages has not been reported. To investigate the regulatory role of MPC1 in LPS-mediated sepsis *in vitro*, we treated RAW264.7 cells with LPS to establish a macrophage sepsis model, monitoring the protein expression of MPC1 at various times post-stimulation. As indicated in Fig. 1A, MPC1 expression decreased significantly 3 h after LPS treatment. Subsequently, we developed an endotoxin tolerance model in RAW264.7 cells and observed that MPC1 expression in the endotoxin-tolerant (ET) group was significantly higher compared to the non-endotoxin-tolerant (LPS) group (Fig. 1B).

Further, we established in vivo sepsis models in mice through either intraperitoneal injection of 4 mg/kg LPS or cecal ligation and perforation (CLP). We detected the expression of MPC1 in peritoneal macrophages (PMs), Kupffer cells (KCs) and bone marrow-derived macrophages (BMDMs) at 0 h, 1 h, 2 h, 3 h, 4 h, 5 h and 6 h after LPS injection or after CLP operation. The *in vivo* results closely mirrored our *in vitro* findings, with significant reductions in MPC1 expression observed 3 h post-LPS injection or CLP (Fig. 1C, D). Additionally, mice that were pretreated with an intraperitoneal injection of 400 μg/kg LPS for 6 h, followed by treatment with 4 mg/kg LPS to induce endotoxin tolerance, exhibited significantly higher MPC1 expression in PMs compared to those in the LPS-only group (Fig. 1E). These findings suggest that the expression of MPC1 in macrophages is suppressed in the sepsis models *in vivo* and *in vitro*.

Overexpression of MPC1 inhibits LPS-mediated oxidative stress and inflammatory response

It is well-documented that lipopolysaccharide (LPS) triggers both oxidative stress and inflammatory responses in monocytes/macrophages[24, 25]. To further investigate the potential modulatory effects of MPC1 on LPS-mediated inflammatory response and oxidative stress, we overexpressed MPC1 in RAW264.7 cells with lentivirus. As depicted in Fig. 2A and 2B, both nucleic acid and protein levels of MPC1 were significantly elevated 72 h after lentiviral transduction. As shown in the Fig. 2C, compared with LPS group, overexpression of MPC1 inhibited the secretion of LPS-mediated pro-inflammatory factors TNF-α and IL-6, indicating that MPC1 inhibited LPS-mediated inflammatory response. Furthermore, we assessed the impact of MPC1 overexpression on oxidative stress markers within these cells. Mitochondrial reactive oxygen species (mtROS) and reactive oxygen species (ROS) serve as key indicators of oxidative stress, with ROS predominantly arising from the transfer of mtROS from mitochondria to the cytoplasm[26]. We found that overexpression of MPC1 also inhibits the production of mtROS and ROS in macrophages, that is, overexpression of MPC1 inhibits LPS-mediated oxidative stress (Fig. 2D, E). Collectively, these results demonstrate that MPC1 possesses anti-inflammatory and antioxidant properties, acting as a negative regulator of both the inflammatory and oxidative stress responses elicited by LPS.

Overexpression of MPC1 does not promote the formation of endotoxin tolerance

Endotoxin tolerance of macrophages can be induced by promoting the expression of anti-inflammatory factors or by inhibiting the expression of pro-inflammatory factors[27, 28]. In the study, we found that MPC1 is a negative regulator of LPS-mediated inflammatory response. In order to study whether MPC1 is involved in the regulation of endotoxin tolerance of macrophages, we categorized RAW264.7 cells into five groups: control (Con), sepsis (LPS), endotoxin tolerance (ET), overexpression of MPC1 with LPS treatment (OE-*MPC1* + LPS), and overexpression of MPC1 in the endotoxin tolerance (OE-*MPC1* + ET). As shown in Fig. 3, compared with LPS group, overexpression of MPC1 inhibited the secretion of TNF-α and IL-6 mediated by LPS (LPS *vs* OE-*MPC1* + LPS). Surprisingly, compared with the ET group, the levels of TNF-α and IL-6 in the OE-*MPC1* + ET group were significantly increased (Fig. 3). These results indicate that overexpression of MPC1 does not promote, and may in fact inhibit, the formation of endotoxin tolerance in macrophages.

Mitochondrial oxidative stress resistance promotes endotoxin tolerance

Mitohormesis describes the adaptation of mitochondria to continuous stress following a mild dose of stress stimulation, providing both mitochondrial and cellular protection. It plays a critical role in regulating a variety of physiological and pathological processes, including aging and cancer [12, 13]. Our previous research demonstrated that TEPP-46, a small molecule agonist of PKM2, promotes mitochondrial biogenesis by facilitating the formation of the PKM2 tetramer, subsequently inducing endotoxin tolerance in macrophages[9]. Similar to mitochondrial biogenesis, mitochondrial oxidative stress resistance is another biological marker of mitohormesis[14]. Whether mitochondrial oxidative

stress resistance is involved in the regulation of endotoxin tolerance needs further study. Mitochondrial oxidative stress resistance refers to the adaptation of mitochondria to re-oxidative stress stimulation after mild dose of oxidative stress stimulation[14]. Mitochondrial oxidative stress resistance is an important endogenous protective way to limit the over-activation of mitochondria by oxidative stress stimulation, mainly reflected in the production of less mtROS and ROS when stimulated by re-oxidative stress. Although mtROS is considered as a pro-inflammatory mediator, it is also an important factor to induce mitochondrial oxidative stress resistance[14]. Inhibition of mtROS production will inhibit mitochondrial oxidative stress resistance, and then expand the inflammatory response when stimulated by secondary oxidative stress[14]. We found that the levels of mtROS and ROS in RAW264.7 cells were significantly increased with LPS treatment, suggesting that LPS promoted the oxidative stress response of macrophages (Fig. 2D, E). Menadione is an oxidant with obvious cytotoxicity, which can induce oxidative damage of cells[29]. As shown in Fig. 3A-C, with the concentration increased of menadione, menadione obviously induced apoptosis of RAW264.7 cells and promoted the expression of ROS. We found that compared with the RAW264.7 stimulated by menadione alone, cells stimulated by LPS for 6 h and then stimulated by menadione can effectively inhibit the production of ROS and significantly improve the cell activity (Fig. 3D, E). This shows that LPS pre-treatment may prime mitochondrial oxidative stress resistance, thus attenuating the oxidative stress response upon subsequent exposure. To further investigate the impact of mitochondrial oxidative stress resistance on endotoxin tolerance, we utilized mito-TEMPO, a selective scavenger of mtROS targeting mitochondria[30]. As shown in Fig. 3F, G, RAW264.7 cells pretreated with mito-TEMPO and then stimulated with LPS can significantly inhibit the production of mtROS mediated by LPS and inhibit mitochondrial oxidative stress resistance.Additionally, compared to untreated cells, the removal of mtROS by mito-TEMPO led to a decreased formation of endotoxin tolerance (Fig. 3H). These findings underscore the importance of mitochondrial oxidative stress resistance in promoting endotoxin tolerance in macrophages.

Overexpression of MPC1 inhibits endotoxin tolerance by inhibiting mitochondrial oxidative stress resistance

The role of MPC1 in regulating mitochondrial oxidative stress resistance in macrophages remains unclear. In this study, we observed that the expression of MPC1 decreased and the levels of mitochondrial reactive oxygen species (mtROS) increased in LPS-treated macrophages (Fig. 1A and Fig. 2D). When MPC1 was overexpressed and cells were stimulated with LPS, we found that overexpression of MPC1 significantly decreased the levels of mtROS and ROS (Fig. 2D, E). These results indicated that overexpression of MPC1 inhibited LPS-mediated oxidative stress. To further explore the impact of MPC1 on mitochondrial oxidative stress resistance, cells were categorized into four groups: control, menadione, LPS + menadione, and OE-*MPC1* + LPS + menadione. We found that the ROS level in OE-*MPC1* + LPS + menadione group was significantly higher than in LPS + menadione group, suggesting that MPC1 overexpression reduced the oxidative stress resistance of macrophages to menadione (Fig. 5A). This implies that MPC1 acts as a negative regulator of mitochondrial oxidative stress resistance.

As we know that overexpression of MPC1 inhibited the formation of endotoxin tolerance in macrophages (Fig. 3). In order to further study whether MPC1 regulates endotoxin tolerance of macrophages by regulating mitochondrial oxidative stress resistance. We applied Timblin's method to induce oxidative stress resistance of RAW264.7 with 4-OHE1[14]. As shown in Fig. 5B, the level of mtROS in RAW264.7 cells increased significantly after being stimulated with 4-OHE1, which inhibited menadione-mediated oxidative stress (Fig. 5C). This confirmed that 4-OHE1 successfully induces mitochondrial oxidative stress resistance.Subsequently, the experiment was expanded to include several groups: control, LPS, endotoxin tolerance (ET), overexpressed MPC1 in the ET (OE-*MPC1* + ET), and 4-OHE1 treatment alongside overexpressed MPC1 in the ET (4-OHE1 + OE-*MPC1* + ET). The findings showed that TNF-α and IL-6 levels in the OE-MPC1 + ET group were significantly elevated compared to the ET group, while in the 4-OHE1 + OE-MPC1 + ET group, these levels significantly decreased, suggesting that inducing mitochondrial oxidative stress resistance with 4-OHE1 could mitigate the suppressive effect of MPC1 overexpression on endotoxin tolerance (Fig. 5D). In summary, these results demonstrate that MPC1 is a negative regulator of LPS-mediated acute inflammation, and its overexpression can effectively reduce LPS-induced inflammatory responses. However, because overexpression of MPC1 also inhibits mitochondrial oxidative stress resistance, it consequently restricts the formation of endotoxin tolerance, substantially diminishing the capacity of macrophages to withstand secondary high-dose LPS stimulation.

SIRT3/SOD2 axis is involved in the regulation of mitochondrial oxidative stress resistance and endotoxin tolerance

SIRT3 and SOD2 are main located in mitochondria, the former has deacetylase activity, and the latter is mitochondrial $O2^-$ scavenger, which mainly removes mtROS[31–33]. It is reported that SIRT3 can induce the deacetylation of SOD2, and participate in the development of many diseases by reducing the level of acetylated SOD2[34]. In LPS-mediated macrophage sepsis model, we found that SIRT3 expression decreased and SOD2 acetylation level increased (Fig. 6A). It is reported that the higher the acetylation level of SOD2, the lower the activity of SOD2 and the weaker the ability to scavenge mtROS[35]. Therefore, we speculate that this may be the main pathway for LPS to promote the production of mtROS. In order to test the above hypothesis, we overexpressed RAW264.7 cells SIRT3 with lentivirus. As shown in Fig. 6B, C, the protein and nucleic acid expression of SIRT3 in RAW264.7 cells increased significantly after 72 h transfection with lentivirus. Besides, overexpression of SIRT3 inhibited the promotion of LPS on macrophage mtROS and ROS, indicating that overexpression of SIRT3 inhibited LPS-mediated oxidative stress (Fig. 6D, E). Then, we established a macrophage sepsis model, and stimulated the cells with menadione, and found that the oxidative stress resistance of macrophages to menadione was weakened (Fig. 6F). 3-TYP is a selective inhibitor targeting SIRT3[36]. After macrophages was stimulated with 3-TYP, the expression of SIRT3 decreased, the acetylation level of SOD2 increased, and mtROS increased (Fig. 6G, H). Compared with using menadione alone, pretreatment with 3-TYP and then treatment with menadione can significantly reduce the cytotoxicity of menadione (Fig. I). This suggests

that SIRT3/SOD2 axis is the key way to mediate mitochondria oxidative stress resistance of macrophage.

Next, In order to further study the regulation of SIRT3/SOD2 axis on endotoxin tolerance, we constructed endotoxin tolerance and endotoxin-intolerant models of RAW264.7 cells. Compared with endotoxin-intolerant group, the expression of SIRT3 in endotoxin-tolerant group increased and the acetylation level of SOD2 decreased (Fig. 6J). Overexpression of SIRT3 inhibits the secretion of TNF-α and IL-6 (Fig. 6K). On the contrary, pretreatment of cells with 3-TYP inhibited the expression of SIRT3 and promoted the release TNF-α and IL-6 (Fig. 6K). Overexpression of macrophage SIRT3, and then established the endotoxin tolerance model of macrophage. We found that overexpression of SIRT3 inhibited the formation of endotoxin tolerance (Fig. 6L). Collectively, these results suggest that SIRT3/SOD2 axis is the key way to mediate mitochondria oxidative stress resistance and endotoxin tolerance of macrophage.

MPC1 regulates mitochondrial oxidative stress resistance and endotoxin tolerance through the SIRT3/SOD2 axis

Our study revealed that under LPS stimulation, the expression levels of MPC1 and SIRT3 decreased, while the acetylation level of SOD2 increased in RAW264.7 cells. In order to further study whether MPC1 regulates the mitochondria oxidative stress resistance and endotoxin tolerance through SIRT3/SOD2 axis, we overexpressed MPC1 with lentivirus, and found that after overexpression of MPC1, the expression of SIRT3 increased and the acetylation level of SOD2 decreased (Fig. 7A). Additionally, suppressing SIRT3 expression with 3-TYP after overexpressing MPC1 led to an increased acetylation level of SOD2 (Fig. 7B). Our experiments demonstrated that MPC1 overexpression reduced the oxidative stress resistance of macrophages to menadione (Fig. 5A). On the basis of overexpression of MPC1, inhibiting the expression of SIRT3 with 3-TYP can increase the oxidative stress resistance of macrophages to menadione and promote the formation of endotoxin tolerance of macrophages (Fig. 7C, D). This suggests that MPC1's modulation of mitochondrial oxidative stress resistance and endotoxin tolerance operates significantly through its effects on the SIRT3 pathway. In Fig. 5A we found that overexpression of MPC1 promotes the expression of SIRT3. Interestingly, overexpression of SIRT3 also promotes the expression of MPC1 (Fig. 7E). However, co-immunoprecipitation (Co-IP) experiments indicated no direct protein interaction between MPC1 and SIRT3 (Fig. 7F), suggesting that the regulatory relationship between MPC1 and SIRT3 may be mediated by other mechanisms or signaling pathways, which warrants further investigation.

Discussions

Sepsis remains a significant global public health challenge, with nearly 50 million new cases annually, contributing to approximately 10 million deaths or about 20% of all global fatalities[37]. Macrophage endotoxin tolerance has been proved to plays an important role in regulating the progress of sepsis[38].During the acute inflammatory response stage, endotoxin tolerance acts as a crucial

regulatory mechanism to prevent tissue damage from overactive inflammatory responses, which can lead to sepsis syndrome[39]. However, prolonged immune tolerance may facilitate secondary infections, increasing mortality, especially in immunocompromised individuals[40]. While some molecular mechanisms of endotoxin tolerance in macrophages have been elucidated, many aspects remain unclear.

Endotoxin tolerance in macrophages is linked to multiple signal transduction pathways. For instance, inhibition of Drp1/Fis1-dependent mitochondrial fragmentation has been shown to enhance macrophage function and immune response in both *in vitro* and *in vivo* models of endotoxin tolerance[8]. Additionally, endotoxin tolerance is closely associated with AMPK-mediated metabolic reprogramming[41] Our research has further defined some regulatory mechanisms underlying macrophage endotoxin tolerance: PKM2 tetramer formation promotes mitochondrial biogenesis, reducing the secretion of inflammatory factors and mitigating organ damage during sepsis[9]; Bone marrow stromal cells can alleviate LPS-induced acute liver injury in mice by inhibiting NLRP3 inflammatory bodies in prostaglandin E 2-dependent KCs[42]; and c-Myb controls TBK1-mediated KCs endotoxin tolerance by negatively regulating DTX4[43]. This study primarily focuses on the role of MPC1 in regulating sepsis and endotoxin tolerance in macrophages.

MPC1 is a protein located on mitochondrial membrane, and its main function is to transport pyruvate located in cytoplasm to mitochondria for metabolic processes[16]. Beyond its roles in tumor formation and aging, MPC1 is also vital in regulating inflammation and immune responses[14]. However, it is not clear whether MPC1 is involved in the regulation of endotoxin tolerance. In this study, we first explored the expression of MPC1 in macrophages in the model of sepsis or endotoxin tolerance *in vitro* and *in vivo*, and found that the protein expression of MPC1 decreased significantly after 3 h of LPS treatment. The level of ROS in cytoplasm reflects the degree of oxidative stress of macrophages[44]. We found that the level of ROS increased significantly when LPS stimulated for 2 h. Studies have shown that endotoxin tolerance of macrophages can be induced by promoting the expression of anti-inflammatory factors or inhibiting the expression of most pro-inflammatory factors[27, 28] Then, we overexpressed MPC1 of RAW264.7 cells with lentivirus. Surprisingly, overexpression of MPC1 can inhibit oxidative stress and inflammatory reaction mediated by LPS, but it does not promote the formation of endotoxin tolerance of macrophages. This suggests that MPC1 overexpression diminishes macrophages' resistance to secondary high-dose LPS stimulation.

We discovered that mitochondrial oxidative stress resistance, a component of mitohormesis, is critical for MPC1's regulation of endotoxin tolerance. Mitohormesis, which includes processes like mitochondrial biogenesis and oxidative stress resistance, serves as a protective mechanism against mitochondrial over-activation[14]. Although mtROS typically act as pro-inflammatory mediators, they also play a crucial role in inducing mitochondrial oxidative stress resistance.[14]. Inhibition of mtROS production will inhibit mitochondrial oxidative stress resistance, and then expand the inflammatory response when stimulated by oxidative stress again[14]. In this study, we found that the levels of mtROS and ROS in RAW264.7 cells increased under LPS stimulation, suggesting that LPS promoted the

oxidative stress response of macrophages. Pre-stimulation of RAW264.7 cells with LPS and then stimulation with menadione can inhibit the cytotoxicity of menadione. These results indicates that LPS pre-stimulation induces mitochondria oxidative stress resistance of macrophages, thus weakening the oxidative stress response when stimulated by oxidative stress again. RAW264.7 cells were cleared of mtROS by mito-TEMPO, which can inhibit the production of mtROS mediated by LPS, inhibit the mitochondrial oxidative stress resistance, and also inhibit the formation of endotoxin tolerance. Furthermore, overexpression of MPC1 not only inhibited the mitochondrial oxidative stress resistance, but also inhibited the formation of endotoxin tolerance of macrophages.

In mechanism, we found that MPC1 regulates mitochondrial oxidative stress resistance through SIRT3/SOD2 pathway, and then regulates macrophage endotoxin tolerance. Overexpression of MPC1 increased SIRT3 expression and decreased SOD2 acetylation. On the basis of overexpression of MPC1, inhibiting the expression of SIRT3 with 3-TYP can improve the acetylation level of SOD2. Interestingly, we found that overexpression of SIRT3 in RAW264.7 cells can also promote the expression of MPC1. researches have confirmed the interaction between SIRT3 and MPC1: Berberine inhibits the interaction between SIRT3 and MPC1, promotes the acetylation of MPC1, induces the degradation of MPC1, and then inhibits gluconeogenesis[45]. SIRT3 binds to the acetylation sites K45 and K46 of MPC1 and inhibits the growth of colon cancer cells[46]. However, in this study, our Co-IP experiments revealed no direct protein interaction between MPC1 and SIRT3. So, how to achieve mutual regulation between MPC1 and SIRT3 remains to be further studied.

Declarations

Author contributions

All authors contributed to the study conception Conceptualization: ZY and LX. Methodology: YL, FQ, and HZ. Investigation: WZ, JG, YL, LX and ZY. Visualization: DH and YZ. Writing: YL and FQ. review: ZY and LX. Editing: YZ, YL and ZY. Supervision: ZY and JG. Funding acquisition: ZY and WZ. All authors contributed to the article and approved the submitted version.

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Availability data and material

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Consent for publication Not applicable

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Conflict of interest The authors declared no competing interest.

Ethical approval The animal study was reviewed and approved by The Research Ethics Committee of Chongqing University Three Gorges Hospital.

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Figures

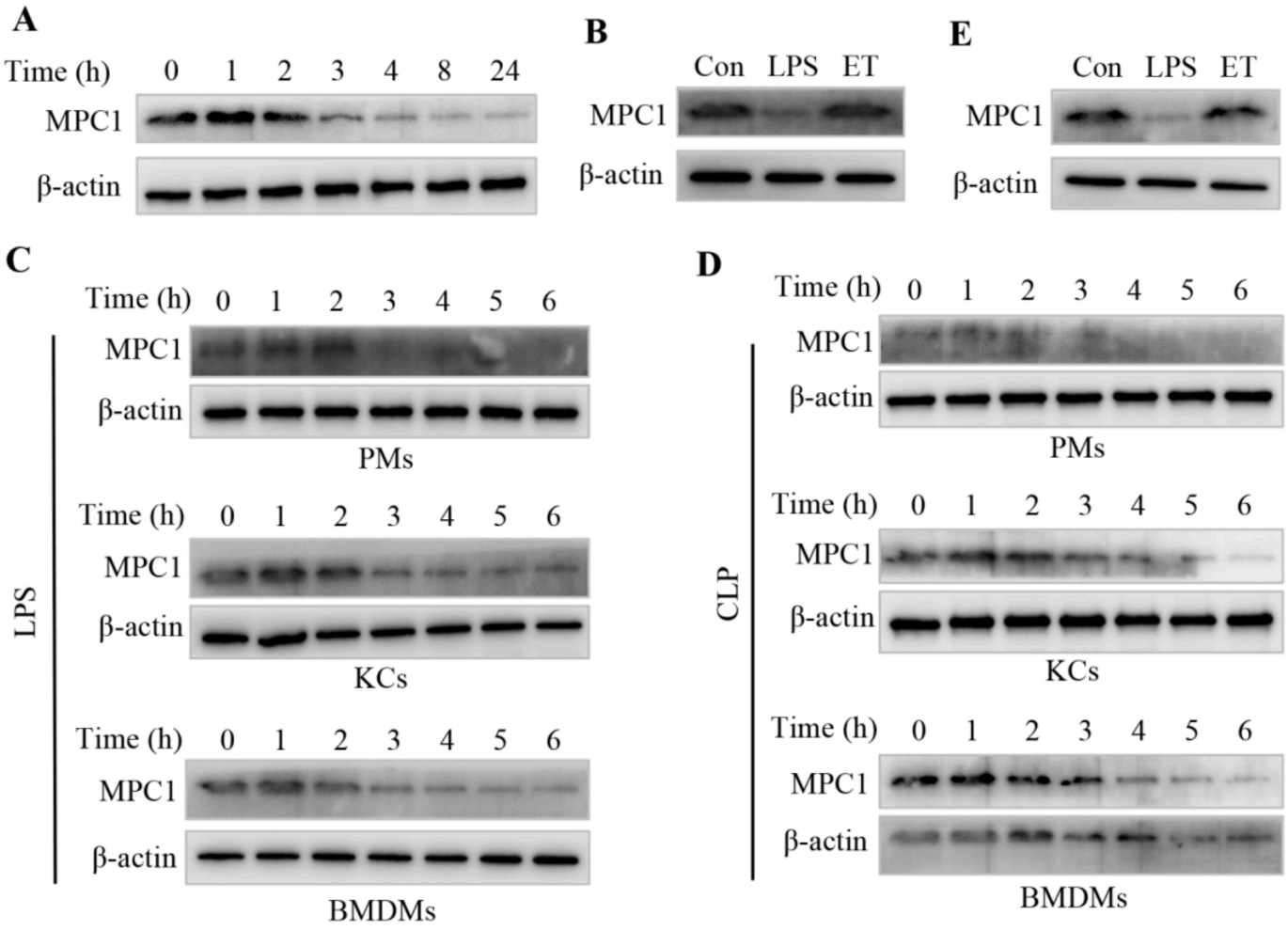

Figure 1

Expression of MPC1 in sepsis model and endotoxin tolerance model. Western blot was used to detect the expression of MPC1 in **(A)** RAW264.7 cells after LPS treated for different time.**(B)** in sepsis model and endotoxin tolerance model *in vitro*. **(C)**in PMs, KCs and BMDMs in mice sepsis model established by intraperitoneal injection of LPS. **(D)** in PMs, KCs and BMDMs in mice sepsis model established by CLP. **(E)** in sepsis model and endotoxin tolerance model *in vivo*.

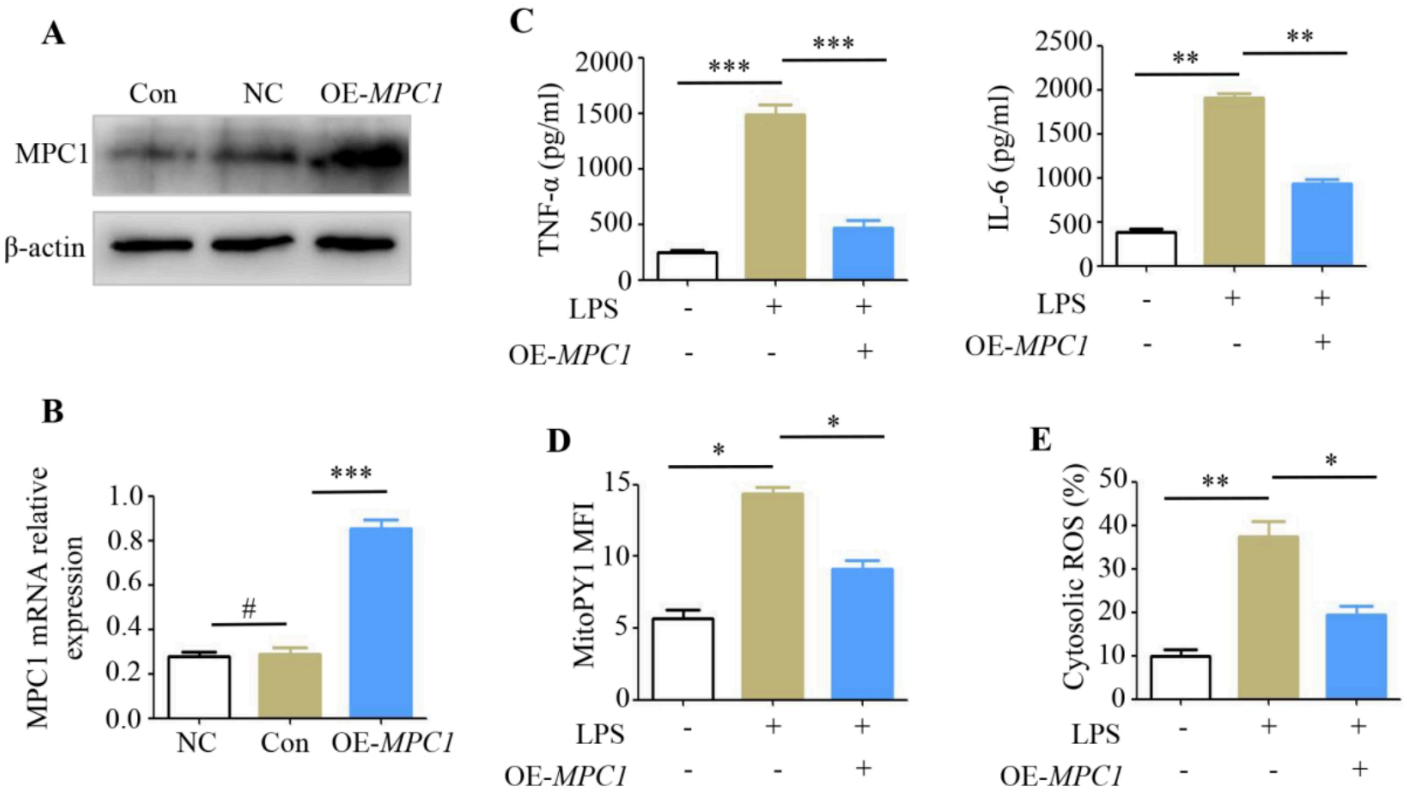

Figure 2

Effect of overexpression of MPC1 on LPS-mediated macrophage activation. **(A)** Western blot was used to detect the transfection efficiency of lentivirus. **(B)** RT-qPCR was used to detect the transfection efficiency of lentivirus. **(C)** Supernatant TNF-a and IL-6 levels in RAW264.7 cells were measured using ELISA. **(D)** Flow cytometry was used to detect the level of mtROS in cells by mitoPY1 probe. **(E)** Flow cytometry was used to detect the level of ROS in cells by CellROX probe. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

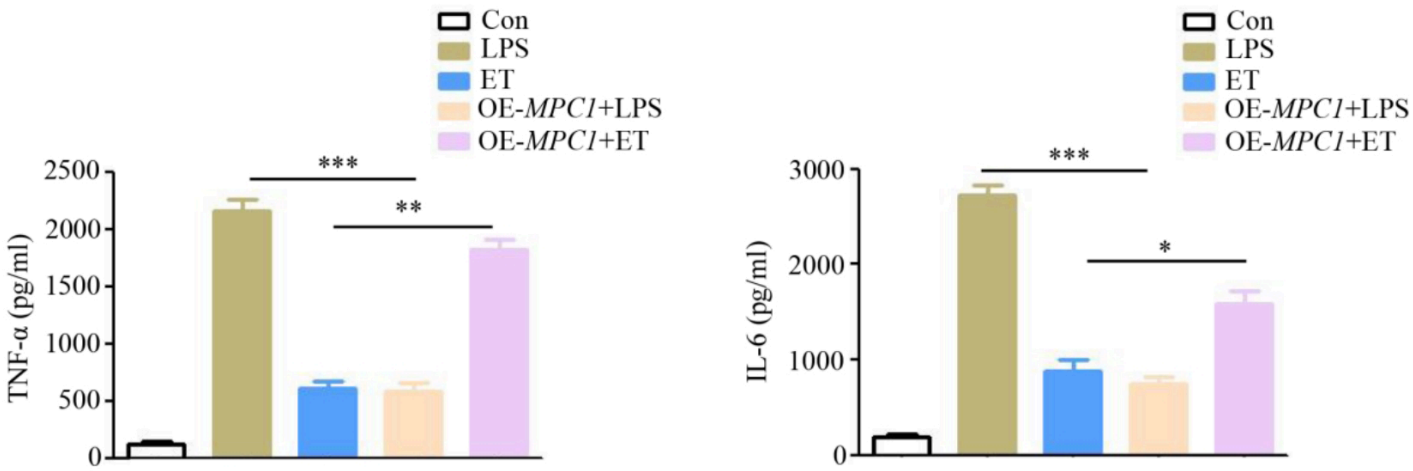

Figure 3

Effect of overexpression of MPC1 on endotoxin tolerance of macrophages. Supernatant TNF-a and IL-6 levels in RAW264.7 cells were measured by using ELISA. *p< 0.05, **p < 0.01, ***p < 0.001.

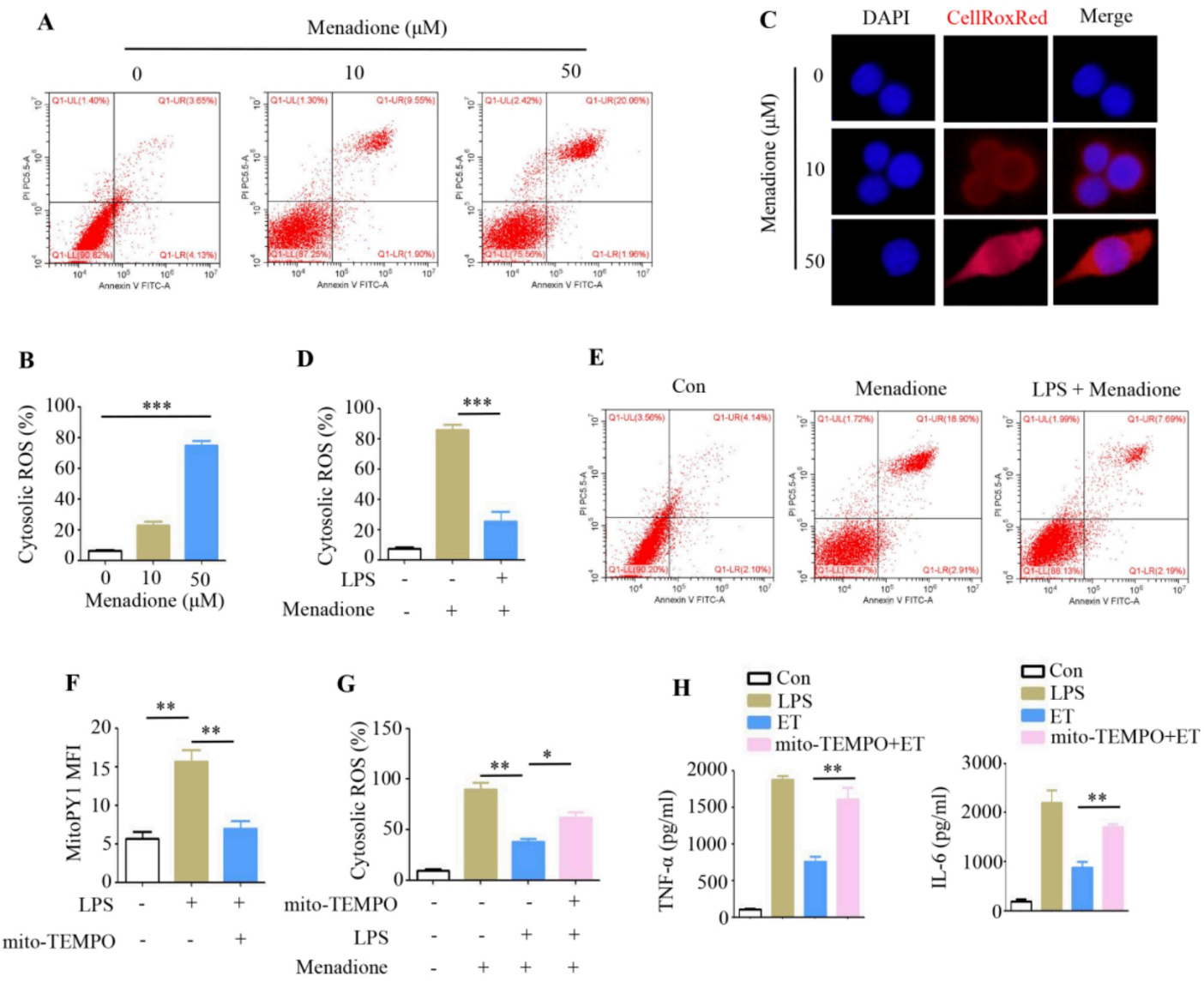

Figure 4

Mitochondrial oxidative stress resistance promotes endotoxin tolerance. **(A)** Cell viability of RAW264.7 cells stimulated with different concentration of menadione was measured using CCK8. **(B)** After cells were stimulated by menadione with different concentrations, ROS levels were detected by flow cytometry. **(C)** The expression of CellROXRed was detected by immunofluorescence with different concentration of menadione treatment. **(D)** Detect the level of ROS. **(E)** Flow cytometry was used to detect the cell viability. **(F)** Flow cytometry was used to detect the level of mtROS in cells by mitoPY1 probe. **(G)**Detect the level of ROS. **(H)** TNF-a and IL-6 levels in supernatant of RAW264.7 cells were measured by using ELISA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

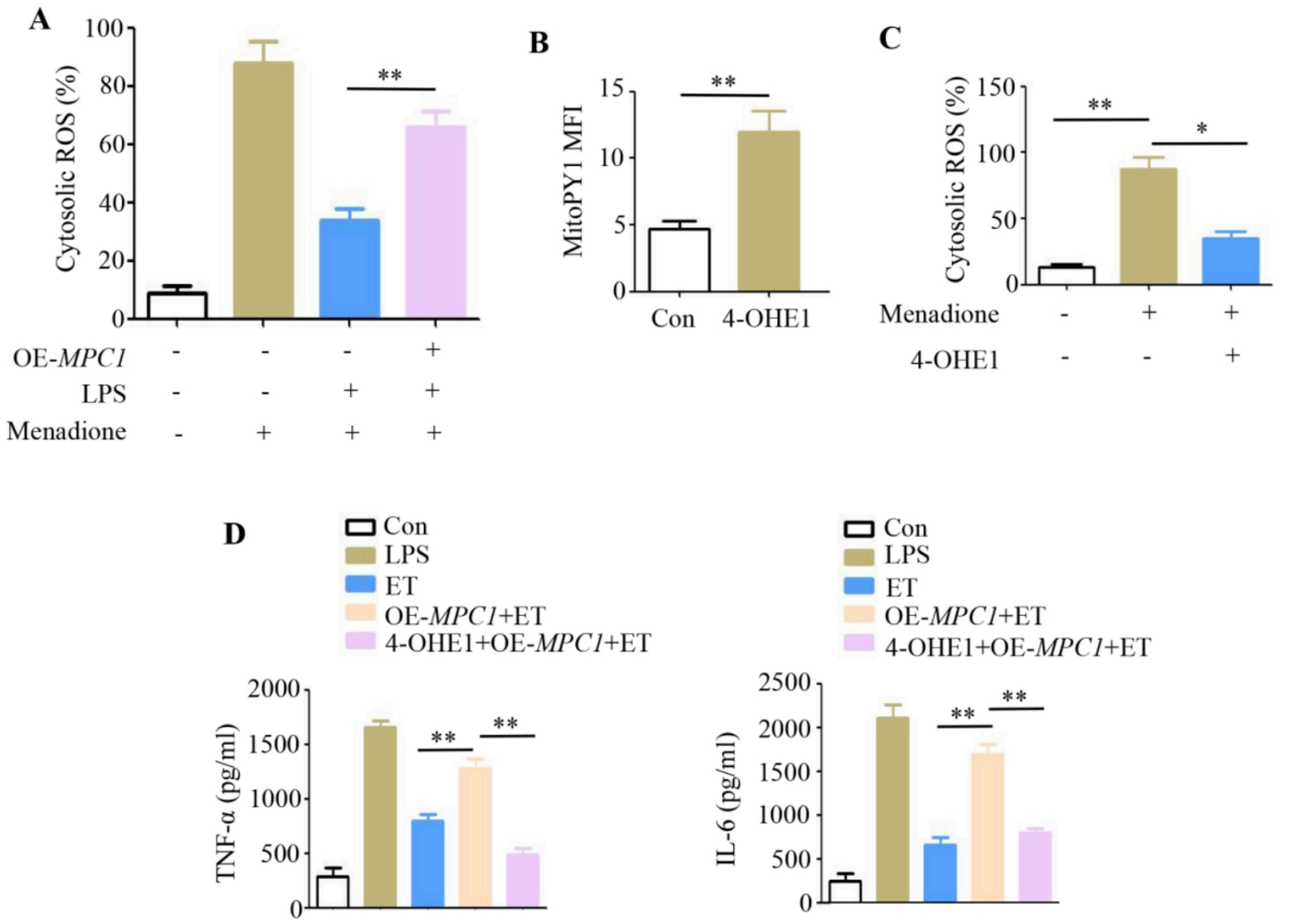

Figure 5

Mitochondrial oxidative stress resistance is required for MPC1 to regulate endotoxin tolerance. **(A)** After the cells overexpressed MPC1, flow cytometry was used to detect the level of ROS in cells. **(B)** After the cells were treated with 4-OHE1, the level of mtROS was detected by flow cytometry. **(C)** Detect the level of ROS. **(D)**TNF-a and IL-6 levels in supernatant of RAW264.7 cells were measured by ELISA. *$p < 0.05$, **$p < 0.01$.

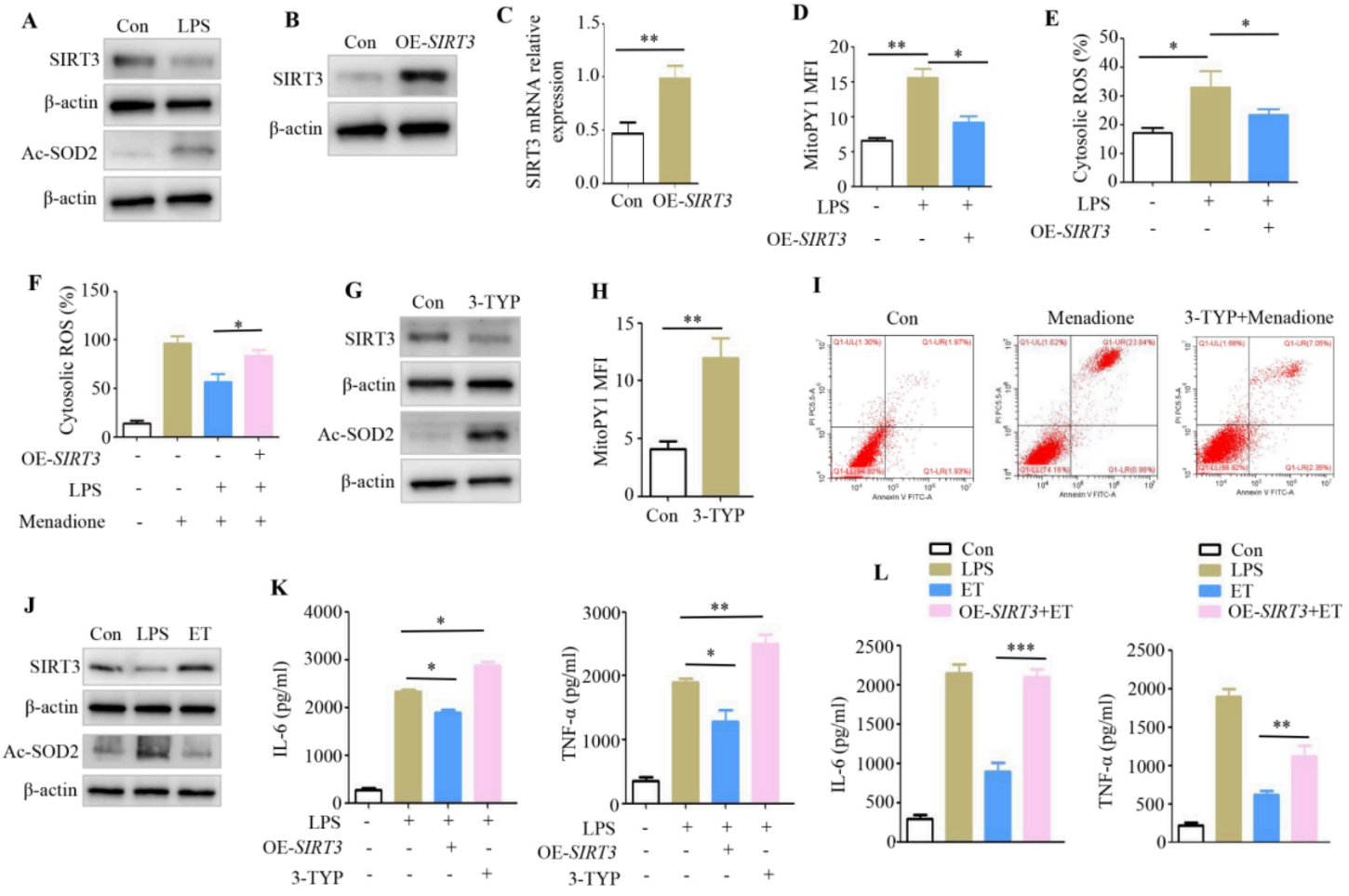

Figure 6

SIRT3/SOD2 axis is involved in regulating mitochondrial oxidative stress resistance and endotoxin tolerance. **(A)**Western blot was used to detect the expression of SIRT3 and SOD2 in cells stimulated by LPS. **(B-C)** Western blot and RT-qPCR were used to detect the transfection efficiency of lentivirus. **(D)** Flow cytometry was used to detect the level of mtROS in cells by mitoPY1 probe. **(E-F)** Detect the level of ROS. **(G)** Western blot was used to detect the expression of SIRT3 and SOD2 in cells stimulated by 3-TYP. **(H)** Detect the level of mtROS. **(I)** Cell viability of RAW264.7 cells stimulated with or without 3-TYP was measured using CCK8. **(J)** Western blot was used to detect the expression of SIRT3 and SOD2 in sepsis model and endotoxin tolerance model *in vitro*. **(K-L)** TNF-a and IL-6 levels in supernatant of RAW264.7 cells were detected by ELISA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

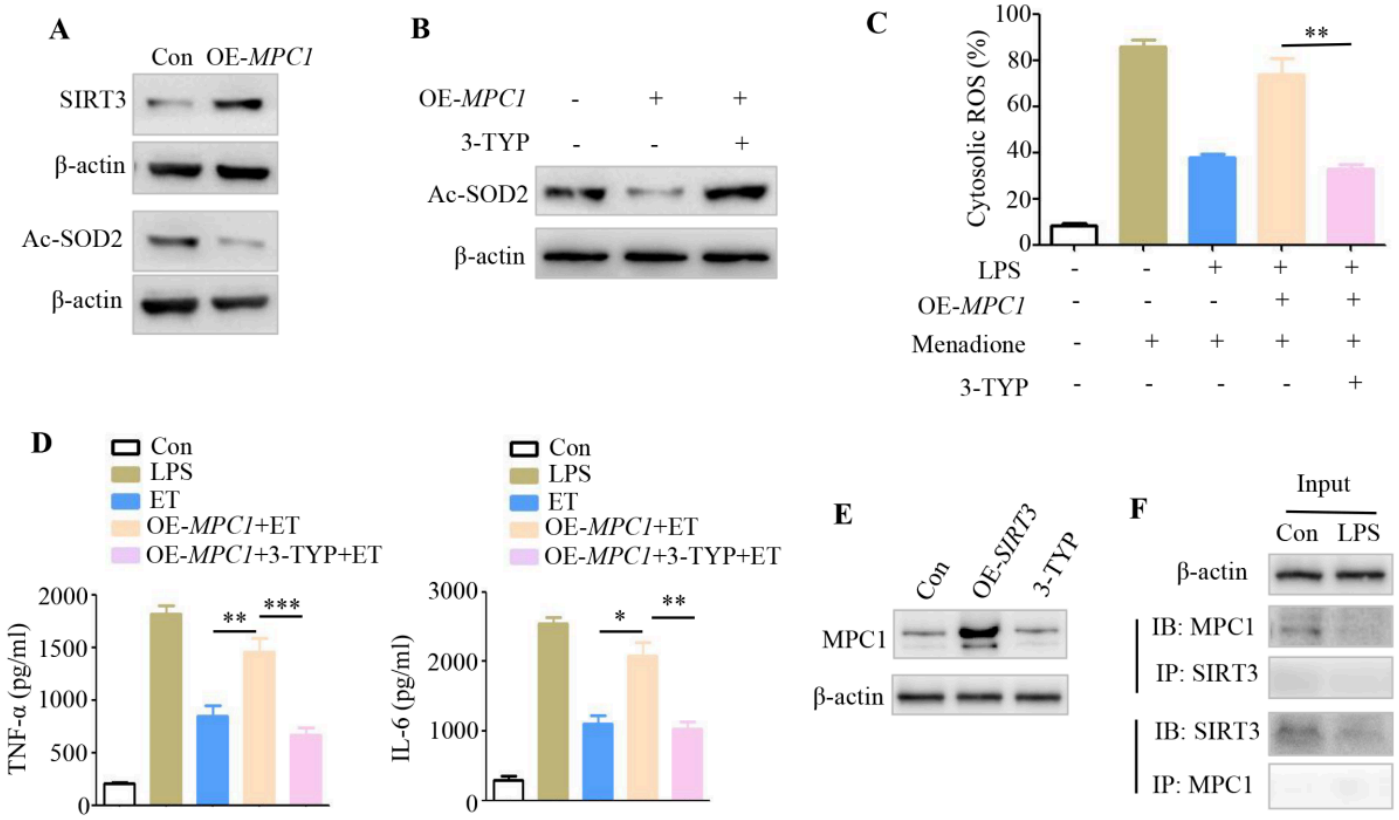

Figure 7

SIRT3/SOD2 axis is the key pathway for MPC1 to regulate mitochondrial oxidative stress resistance and endotoxin tolerance. **(A)** Western blot was used to detect the expression of SIRT3 and SOD2 in cells after overexpression of MPC1. **(B)**Western blot was used to detect the level of SOD2. **(C)** Detect the level of ROS. **(D)** TNF-a and IL-6 levels in supernatant were detected by ELISA. **(E)** Western blot was used to detect the expression of MPC1 in cells after overexpression of SIRT3. **(F)** Co-IP was used to detect the interaction between MPC1 and SIRT3. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.