International Immunopharmacology 73 (2019) 471–481

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Dexmedetomidine alleviates LPS-induced apoptosis and inflammation in macrophages by eliminating damaged mitochondria via PINK1 mediated mitophagy

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A B S T R A C T

The macrophage is an innate immune response cell that plays an important role in the development of sepsis. Dexmedetomidine (DEX) is a sedation drug, which have anti-oxidative, anti-inflammatory and anti-apoptosis effects and can be used on sepsis patients in the ICU. However, its mechanisms of action remain poorly understood. PTEN-induced putative kinase 1 (PINK1) is a mitochondrial serine/threonine protein kinase that recognizes damaged mitochondria and leads to mitophagy. This study investigated the effects of DEX on Lipopolysaccharides(LPS)-induced macrophage injury and explained the underlying mechanisms. The results showed that LPS treatment caused mitochondrial damage, mitochondria-dependent apoptosis and PINK1-mediated mitophagy; at the same time, PINK1 has a protective effect on LPS-induced macrophage apoptosis and inflammation by mitophagy that eliminates dysfunctional mitochondria. DEX could promote the clearance of damaged mitochondria characterized by low Mitochondrial membrane potential (MMP) and high reactive oxygen species(ROS), thus exerting a protective effect in LPS treated macrophages, and PINK1 mediated mitophagy is required for this protective effect.

1. Introduction

Sepsis is a disease caused by a dysregulated host response to infection, and it is a life-threatening organ dysfunction [1]. Epidemiological studies showed that the number of deaths from sepsis is the same as acute myocardial infarction, and it has become the tenth leading cause of death in the United States [2]. As important innate immunoreactive cells, macrophages play an important role in the development of infection [3]. LPS, also known as lipoglycans and endotoxins, are found in the outer membrane of gram-negative bacteria and are typically used to mimic the inflammatory model.

Autophagy is an essential homeostatic process by which cells break down their own components [4]. During autophagy, misfolded or aggregated proteins and dysfunctional cellular organelles are engulfed with a double membraned vesicle, and then the cytoplasmic cargo are delivered to the lysosome for degradation [5]. Mitophagy, the specific autophagic elimination of dysfunctional or damaged mitochondria, is a quality control system that dampens inflammation [6], reduces cell death signals and prevents unwarranted cell loss after cells encounter various stimuli [7]. One simple fact is that PINK1 is intimately involved with mitochondrial quality control by identifying damaged mitochondria and targeting specific mitochondria for degradation through PINK1/parkin-mediated mitophagy [8]. Healthy mitochondria maintain a membrane potential that can import PINK1, where it is subsequently cleaved by PARL. While damaged mitochondria are being recognized by PINK1, PINK1 accumulates on the outer membrane of the mitochondria and recruits parkin, then the mitochondria are designated for degradation by lysosomes through the PINK1/parkin pathway [9].

ROS are products of normal cellular metabolism and are known to act as second messengers under physiological conditions. Meanwhile, the overproduction of ROS, most frequently due to excessive stimulation of either reduced nicotinamide adenine dinucleotide phosphate (NADPH) by pro-inflammatory cytokines, such as tumor necrosis factor-a (TNF-a) and interleukin-1b (IL-1b), results in oxidative stress [10]. The mitochondrial permeability transition pore(mPTP) can then be activated by excessive ROS, thus causing mitochondrial dysfunction [11]. Damaged mitochondria release or display signals, such as mtROS and mtDNA, that promote NLRP3-inflammasome activation and lead to caspase-1 activation [12], while both autophagy/mitophagy and apoptotic cell death tend to dampen inflammation [13,14].

DEX is a sedation drug primarily used in post-anesthesia care and intensive care units for sedation and analgesia. Increasing lines of

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https://doi.org/10.1016/j.intimp.2019.05.027
Received 9 September 2018; Received in revised form 18 January 2019; Accepted 15 May 2019
Available online 03 June 2019

evidence have shown that DEX exerts anti-inflammatory and anti-apoptotic effects and attenuates mitochondrial dysfunction [15–17], while Liu et al. [18] have found that DEX could attenuate the inflammatory reaction by activating cholinergic anti-inflammatory pathways, although some other pathways remain unclear. In this study, we hypothesized that DEX could regulate mitophagy to promote the clearance of damaged/dysfunctional mitochondria, therefore exerting anti-inflammatory and anti-apoptotic effects. The RAW 264.7 cell line was originally a leukemia cell line, but it also exhibits some macrophage functions and is widely used to generate inflammation models [19,20]. To test our hypothesis, we stimulated the RAW 264.7 macrophages with LPS to mimic pathological conditions, and then we treated the LPS-primed macrophages with DEX to explore the effects of DEX on mitophagy, inflammation and apoptosis.

2. Materials and methods

2.1. Reagents

Cell culture medium DMEM (Dulbecco's Modified Eagle Medium, #11965-092), Fetal Bovine Serum (FBS, #10099-141) and cell scrapers (#179693) were purchased from Thermo-Fisher Scientific. LPS (Lipopolysaccharides from *Escherichia coli* O127:B8, #L4516-1MG) was purchased from Sigma (USA). Antibodies against LC3 (#ab192890), parkin (179812), PINK1 (ab186315, ab23707) and TOM20 (186734) were purchased from Abcam (USA), antibody against Beclin1 (ARG54094) was purchased from Arigo (China), antibodies against GAPDH (#60004-1-Ig), cyto-c (10993-1-AP) and caspase-9 (#10380-1-AP) were purchased form Proteintech (USA), antibody against caspase-3 (#9664) was purchased from Cell Signaling Technology (USA), Goat Anti-Mouse IgG (#220-0341) and Goat Anti-Rabbit IgG (#074-1506) were purchased from KPL (USA), DyLight 488, Goat Anti-Mouse IgG (#A23210) and DyLight 549, and Goat Anti-Rabbit IgG (#A23320) were purchased from Abbkine (China). The mitochondrial membrane potential assay kit with JC-1 and the NO assay kit were purchased from Beyotime Biotechnology (China). The ROS assay kit and the ATP assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (China). ELISA kits for IL-1β and TNF-α were purchased from R&D Systems (USA). The Cell Counting Kit-8 was purchased from Dojindo (Japan). The TUNEL assay kit was purchased from GeyGen BioTech (China). The RNApure Tissue & Cell Kit (#CW0584) and cDNA synthesis kit (#CW0742S) were purchased from CWBIO (Beijing, China). The TransStart Greed q-PCR SuperMix (#AQ101) was purchased from Transgen (China).

2.2. Cell culture and treatments

Mouse macrophage RAW 264.7 cells were cultured in DMEM with 10% FBS in a humidified incubator (5% CO2) at 37 °C and dislodged from the flask substrate with a cell scraper when the confluence reached approximately 80%. The subcultivation ratio of 1:3 to 1:6 is recommended, and medium should be replaced approximately every 2 days. RAW 264.7 cells (2×10^5 cells/ml) were randomly seeded into dishes or plates, and the corresponding treatments were given to each group when the confluence reached approximately 60%—70%. The concentration of LPS was 1000 ng/ml, the concentration of dexmedetomidine was 1 μM, the concentration of atipamezole was 10 nM and the control group received the same volume of PBS buffer. Of note, protease inhibitors E-64d (10 μg/ml) and Pepstatin A (10 μg/ml) were recommended to be added into the medium when the cell culture was used for LC3 western blotting, according to the guidelines [21].

2.3. Measurement of cell viability

Cell suspension (100 μl/well) was inoculated in a 96-well plate, and the treatments were given at proper confluence. Twelve hours later, 10 μl of CCK-8 solution was added to each well of the plate for 2 h of incubation, and then we measured the absorbance at 450 nm using a microplate reader.

2.4. Measurement of TNF-α and IL-1β

Cytokine concentrations in the supernatant were determined by ELISA Kit, according to the manufacturer's instructions, and then the absorbance at 450 nm was measured using a microplate reader.

2.5. Measurement of NO production

The nitrite levels in cell culture supernatant were detected using the NO Assay Kit, according to the manufacturer's instructions, and the absorbance was measured by the microplate reader at 550 nm.

2.6. Detection of ROS

The reactive oxygen species assay kit was used to detect the accumulation of ROS in Raw 264.7 cells. Cells were cultured on coverslips in 96-well plates, and the procedures were executed according to the manufacturer's instructions. Fluorescence was examined by a microplate reader with luminometer function.

2.7. Assessment of MMP by JC-1 kit

The mitochondrial membrane potential (MMP) of macrophages was measured by JC-1, an MMP-sensitive fluorescent dye. Cells were cultured on the coverslips in 24-well plates, and the procedures were executed referring to the assay kit. Images were acquired with a fluorescence microscope equipped with a digital camera. The relative MMP was calculated using the ratio of J-aggregate/monomer (590/520 nm).

2.8. Measurement of intracellular ATP

The ATP content was detected by the ATP Assay Kit. The experimental procedure was conducted according to the manufacturer's instructions. The relative light unit was measured by a microplate reader with luminometer function.

2.9. TUNEL assay

The TUNEL assay was used to measure the LPS-induced macrophage apoptosis. The protocol was conducted according to the manufacturer's instructions for the assay kit. Images were acquired with a fluorescence microscope.

2.10. Extraction of proteins

After treatment, RAW 264.7 cells were washed three times with cold phosphate-buffered saline (PBS). We then extracted the protein fractions using RIPA extraction reagent and cocktail (vol/vol, 100:1), and then the protein concentration was determined by the BCA Protein Assay Kit according to the manufacturer's protocol. Protein extracts were used for western blot analysis.

2.11. Western blot analysis

Total protein of RAW 264.7 cells was extracted using the procedures above. Equal amounts of protein lysates were separated by SDS-PAGE (10%, 12%) and transferred onto PVDF (0.45 μm, 0.22 μm) membranes, followed by blocking with 5% skimmed milk at room temperature for 2 h. Subsequently, the membranes were incubated with the primary antibodies at 4 °C overnight. After washing with TBS-T five times, the membranes were incubated with the HRP-conjugated secondary

antibody (1:8000) for 1 h. Western blot bands were imaged by Protein Simple (USA) and analyzed by ImageJ.

2.12. Immunofluorescence

Cells were plated on glass bottom cell culture dishes and treated as indicated, then the cells were fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.3% Triton X-100 for 20 min. After blocking in PBS containing 10% fetal bovine serum for 1 h, cells were incubated overnight with primary antibodies, incubated the next day with secondary antibodies for 1 h, and then the cells were incubated with DAPI to indicate the nucleus. Cells were viewed using a laser-scanning confocal microscope.

2.13. siRNA and plasmid transfections

Short hairpin RNA (shRNA) sequences directed against PINK1 were designed and synthesized by the Shanghai Genepharma Company (Shanghai, China). The sequences were: PINK1 (F) GCACACUGUUCC UCGUUAUTT, (R) AUAACGAGGAACAGUGUGCTT. GAPDH (F) CTTT GGTATCGTGGAAGGACTC, ® GTAGAGGCAGGGATGATGTTCT. For transfection, siRNA or control in Lipofectamine 3000 (Invitrogen) was transfected into cells according to the manufacturer's instructions. After twenty-four hours, cells were harvested for total RNA extraction or treated as indicated.

2.14. Gene expression analyses by RT-qPCR

After 24 h of transfection, total RNA was prepared using the RNApure Tissue & Cell Kit, according to the manufacturer's instructions, and reverse-transcribed into first-strand cDNA using the cDNA synthesis kit. Real-time PCR was performed using TransStart Green q-PCR SuperMix. To normalize mRNA levels, GAPDH was assessed at the same. The $2^{-\triangle\triangle Ct}$ method was used to calculate the relative changes in mRNA expression.

2.15. Transmission electron microscopy

Cells were treated as indicated and then were fixed in 2.5% glutaraldehyde at 4 °C overnight and postfixed with 2% osmium tetroxide for 1.5 h at room temperature. After fixation, cells were embedded and stained with uranyl acetate/lead citrate. The sections were examined under a transmission electron microscope at 60 kV.

3. Statistical analysis

All results are reported as the mean ± SD. Statistical significance was evaluated using one-way analysis of variance or unpaired Student's *t*-test (using GraphPad Prism). P values < 0.05 were considered significant.

4. Results

4.1. Autophagy, inflammation and apoptosis were induced in RAW 264.7 cells during LPS treatment

According to our previous study [22], we used LPS (1000 ng/ml) to stimulate macrophages to mimic the inflammatory model. Yi Xu et al. [20] and our studies (Fig. 1A) have shown LPS-induced autophagy to be maximal at 12–16 h following LPS (1000 ng/ml) stimulation. We thus chose 12 h as the time point, and the dose titration experiments revealed that cell viability was dose-dependent between 50 and 2000 ng/ml (Fig. 1B). It is well known that inflammatory cytokines IL-1β and TNF-α are believed to be critical factors in inflammation [23,24], and so we used ELISA kits to examine the production of IL-1β and TNF-α. Upon LPS treatment, we found that the secretion of IL-1β, TNF-α (Fig. 1G and H) and NO production (Fig. 1E) were increased in comparison with the control group. To investigate the effects of LPS treatment on cell death in macrophages, we used the CCK8 assay kit and western blot. As shown in Fig. 1, cell viability was decreased (Fig. 1D) and the expression of active caspase-3 (Fig. 1C) was increased in the LPS group. Autophagy is a conservatively homeostatic process triggered when cells encounter stress or pathological conditions; sometimes autophagy is called autophagic cell death or type II cell death. Therefore, we examined whether autophagy is induced in this model. As can be seen in Fig. 1, the expression of LC3-II (Fig. 1C) and the numbers of LC3-dots (Fig. 1F) were increased in the LPS group. All of these data revealed that autophagy, inflammation and apoptosis are induced in RAW 264.7 cells upon LPS treatment.

4.2. LPS treatment caused mitochondrial damage and mitochondria-dependent apoptosis

Previous studies have found that mitochondria play an important role in sepsis [25,26], so we were curious about the change of mitochondrial function in LPS-treated macrophages. To examine whether LPS treatment caused mitochondrial dysfunction in macrophages, we used the JC-1 kit, the ATP kit and the ROS kit to measure the levels of MMP, cellular ATP and ROS, respectively. Compared with the control group, the levels of MMP and intracellular ATP were decreased and the ROS level was increased in the LPS group (Fig. 2, B, C and D), indicating mitochondrial dysfunction in macrophages. Furthermore, in order to detect the quantity of mitochondria, we used western blotting to measure the expression of TOM20. As shown in Fig. 2, the expression of TOM20 was decreased after LPS stimulation in comparison with the control group (Fig. 2A), which indicates the loss of mitochondria in the LPS group. It is well known that there exist two distinct death pathways—an extrinsic (death-receptor) pathway and an intrinsic (mitochondrial) pathway [27]. We wondered whether there is any relationship between mitochondrial dysfunction and apoptosis; therefore, we used western blotting to examine the expression of cyt-c and caspase-9, which are two important proteins in the mitochondrial-dependent apoptosis pathway, and we found that the expressions of cyt-c and caspase-9 were increased during LPS treatment (Fig. 2A). All of these results indicated that LPS treatment can cause mitochondrial damage and mitochondria-dependent apoptosis.

4.3. PINK1 accumulates on damaged mitochondria under LPS treatment and promotes mitophagy

High levels of ROS can induce mitochondrial dysfunction, which results in a further increase in ROS levels and eventually leads to cell death [28]. This phenomenon suggests that the removal of dysfunctional mitochondria is necessary for maintaining the redox balance and cell survival. Recently, studies have found that cells could dampen inflammation through mitophagy, reduce cell death signals and prevent unwarranted cell loss after various stimulation [6,7] and that PINK1 protein has played an important role in this process [29]. To verify whether mitochondria morphology changes and mitophagy are induced by LPS stimulation, we used transmission electron microscopy to view the ultrastructure of macrophages, and the results showed that the number of mitophagosomes increased and the mitochondrial morphology changed in the LPS group as compared with the control group (Fig. 3C). We then determined whether the expression of PINK1 and Parkin is changed during LPS stimulation. The results obtained from western blot analysis showed that expression of PINK1 and Parkin increased in the LPS group after mitochondrial dysfunction (Fig. 3A). To determine whether increased PINK1 accumulation in LPS-stimulated macrophages correlates with active mitophagy, we used immunofluorescence staining to view the changes of PINK1 and TOM20. From the immunofluorescence image (Fig. 3C), we can see that the expression of PINK1 increased, while the numbers of mitochondria decreased, as

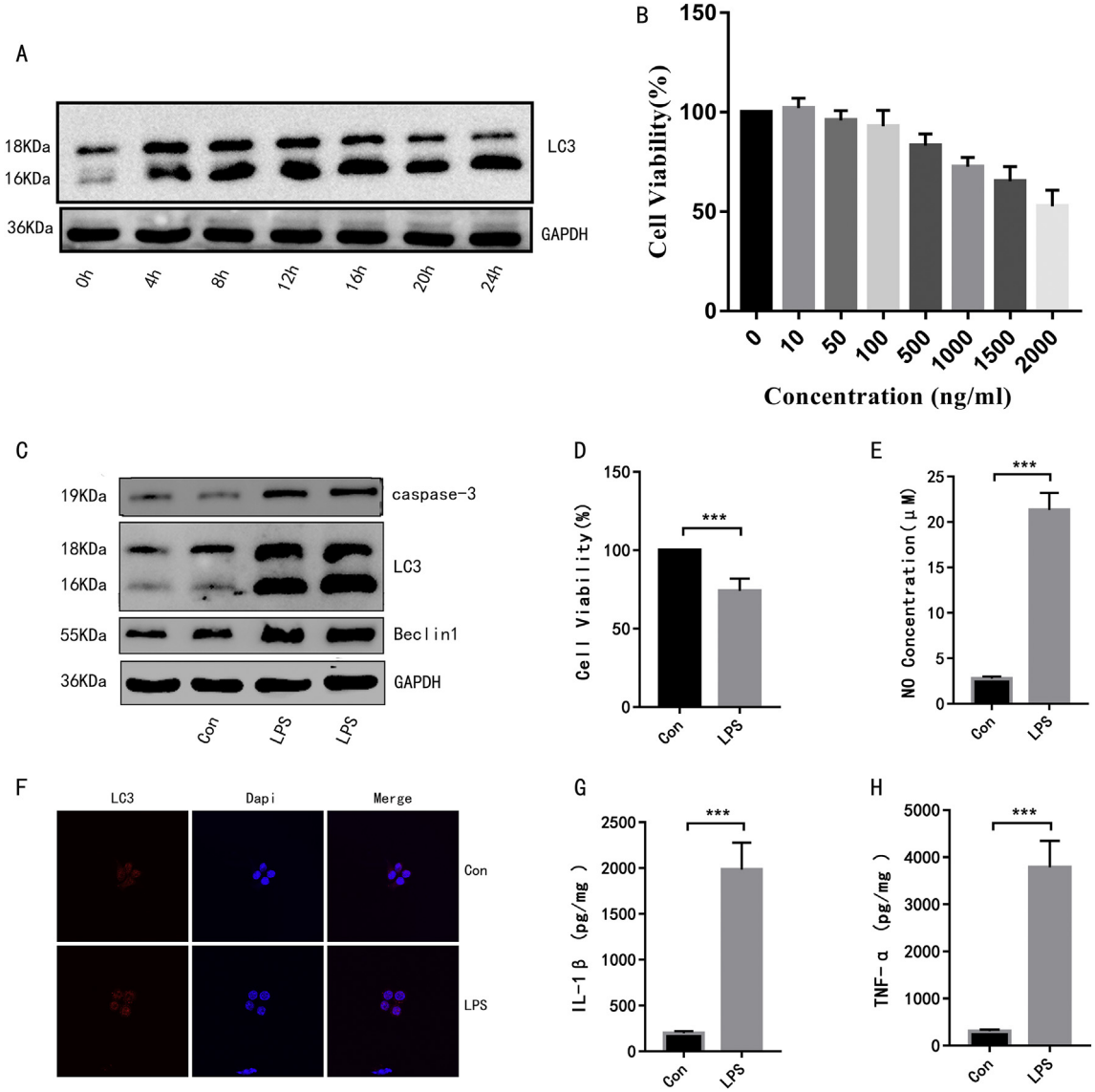

Fig. 1. Autophagy and apoptosis protein expression, cytokines, NO concentration and cell viability changes in macrophages upon LPS stimulation. Raw 264.7 macrophages were stimulated without (con) or with LPS. (A) The expression of LC3 during time course LPS treatment (1000 ng/ml). (B) Cell viability between different concentrations of LPS treatment. (C) Autophagy related proteins LC3 and Beclin1 and apoptotic caspase-3 were analyzed by western blotting. (D) Cell viability was measured by CCK8 assay. (E) NO concentration was measured by the NO Griess assay kit. (F) Quantification of LC3 punctate formation in macrophages measured by immunofluorescence. (G and H) IL-1β and TNF-α secretions were measured by enzyme-linked immunosorbent assay (ELISA). All data were expressed as the mean ± SD.

TOM20 showed in the LPS group; meanwhile, the green and red fusion puncta increased in the merge channel in the LPS group. After PINK1 accumulates on the outer membrane, it will recruit Parkin to the damaged mitochondria and lead to mitophagy. All data presented demonstrated that PINK1 accumulates on damaged mitochondria and promotes mitophagy under LPS treatment.

4.4. The effect of PINK1 on mitochondrial clearance, inflammation, oxidative stress and apoptosis

Accumulation of PINK1 on the outer membranes of damaged mitochondria and active mitophagy are regarded as essential processes for the clearance of dysfunctional mitochondria. We hypothesize that destruction of any process may lead to the accumulation of dysfunctional mitochondria. To test this hypothesis, we used PINK1 siRNA to disrupt the process of mitophagy. The expressions of PINK1 protein and mRNA were significantly reduced in macrophages transfected with PINK1 siRNA in comparison to those transfected with scrambled siRNA (Fig. 4, A and G). Studies have found that under normal conditions, basal mitophagy occurs independently of PINK1 in mouse tissues of high metabolic demand, such as heart, liver and microglia [30], which is in line with our results in macrophages, as the data are comparable between PINK1 wild-type and knockdown cells. Upon LPS stimulation, PINK1 knockdown in macrophages elicited the accumulation of dysfunctional mitochondria in comparison with the siCon + LPS group, as measured by transmission electron microscopy (Fig. 4H), and autophagy was shown to be inhibited as the expression of LC3-II was reduced (Fig. 2A); these results are consistent with those of McWilliams TG that upon stimulation, PINK1 knockdown inhibits the clearance of dysfunctional mitochondria by mitophagy. Meanwhile, the concentrations of IL-1β, TNF-α and the concentration of ROS increased, and cell viability and ATP concentration decreased, as shown in Fig. 4 (B–F). We then used western blotting to examine the effect of PINK1 knockdown on autophagy/mitophagy and apoptosis during LPS stimulation: as shown in

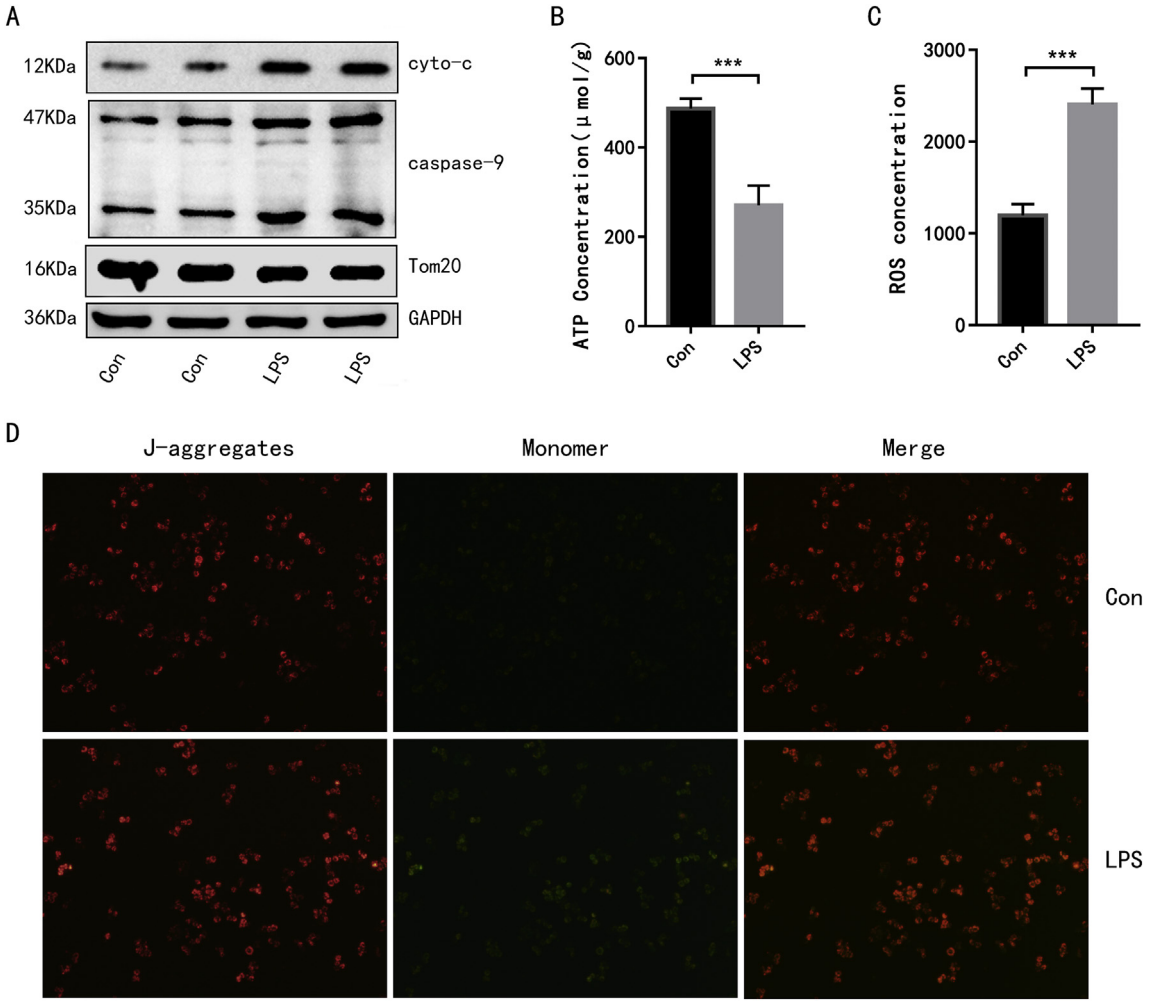

Fig. 2. Mitochondrial function, quantity and mitochondrial-dependent apoptosis protein expression in macrophages upon LPS stimulation. Raw 264.7 macrophages were stimulated without (con) or with LPS (1000 ng/ml) for 12 h. (A) Mitochondrial-dependent apoptosis proteins cyto-c and caspase-9 and TOM20 were analyzed by western blotting. (B) ATP concentration was measured by ATP assay kit. (C) ROS concentration was measured by ROS assay kit with multimode reader. (D) Macrophages were stained with JC-1, and the mitochondrial transmembrane potential (ΔΨm) was measured by fluorescence. All data were expressed as the mean ± SD.

Fig. 4, the expression of LC3-II decreased and the expression of active caspase-3 increased when compared with the siCon+LPS group (Fig. 4A), and these results indicate that knockdown of PINK1 inhibits autophagy/mitophagy and enhances apoptosis during LPS stimulation. Taken together, these results indicated that the clearance of dysfunctional mitochondria by mitophagy is important for the prevention of apoptosis and the accumulation of dysfunctional mitochondria during LPS stimulation, while PINK1 has played an important role in this process and is required for mitochondrial clearance.

4.5. Dexmedetomidine promotes mitophagy, attenuates mitochondrial dysfunction and inhibits mitochondrial-dependent apoptosis

Dexmedetomidine (DEX) is a sedation drug which is widely used for sepsis patients, and studies have found that DEX could attenuate oxidative stress-induced lung alveolar epithelial cell apoptosis and attenuate lipopolysaccharide-induced acute lung injury and apoptosis in rats; however, there have been no such research hypotheses regarding the effects of DEX on macrophages. To detect the protective effects of DEX, we treated the LPS-primed macrophages with DEX to explore the effects of DEX on macrophages. Western blotting showed that the expressions of cyt-c, caspase-9 and active caspase-3 in the LPS + DEX group were decreased in comparison with the LPS group (Fig. 5A), and the TUNEL assay showed fewer TUNEL positive cells in the LPS + DEX group when compared with the LPS group (Fig. 5H), which indicated that DEX could inhibit mitochondrial apoptosis in LPS-treated macrophages. Furthermore, the data from CCK8 assays (Fig. 5B) were consistent with these results. We then used ELISA to verify the protective effects of DEX on cytokine secretions in LPS-treated macrophages, and the results proved that DEX could reduce the secretion of IL-1β and TNF-α (Fig. 5, F and G). Meanwhile, we examined the effects of DEX on cellular ATP and ROS concentrations, finding that DEX exerts a protective effect on mitochondrial function (Fig. 5, C and E). Because DEX is a potent α2 adrenergic agonist, we used a nonspecific antagonist of α2 receptors, atipamezole (Ati), to block the activation of α2 receptors, but it failed to completely reverse the effect of DEX on LPS-treated macrophage, indicating that there must be additional signaling pathways. Therefore, we were curious about whether autophagy/mitophagy is involved in this process. To investigate the effect of DEX on autophagy/mitophagy, we used western blotting and immunofluorescence. The results showed that the expressions of LC3- II (Fig. 5A) and LC3-dots (Fig. 5D) increased, which indicated the promotion of autophagy. In the meantime, the expressions of PINK1 (Fig. 5A) and fusion puncta of LC3 and PINK1 increased in the LPS + DEX group in comparison with the LPS group (Fig. 5D), which means that more mitophagosomes were induced in the LPS + DEX group to eliminate the dysfunctional

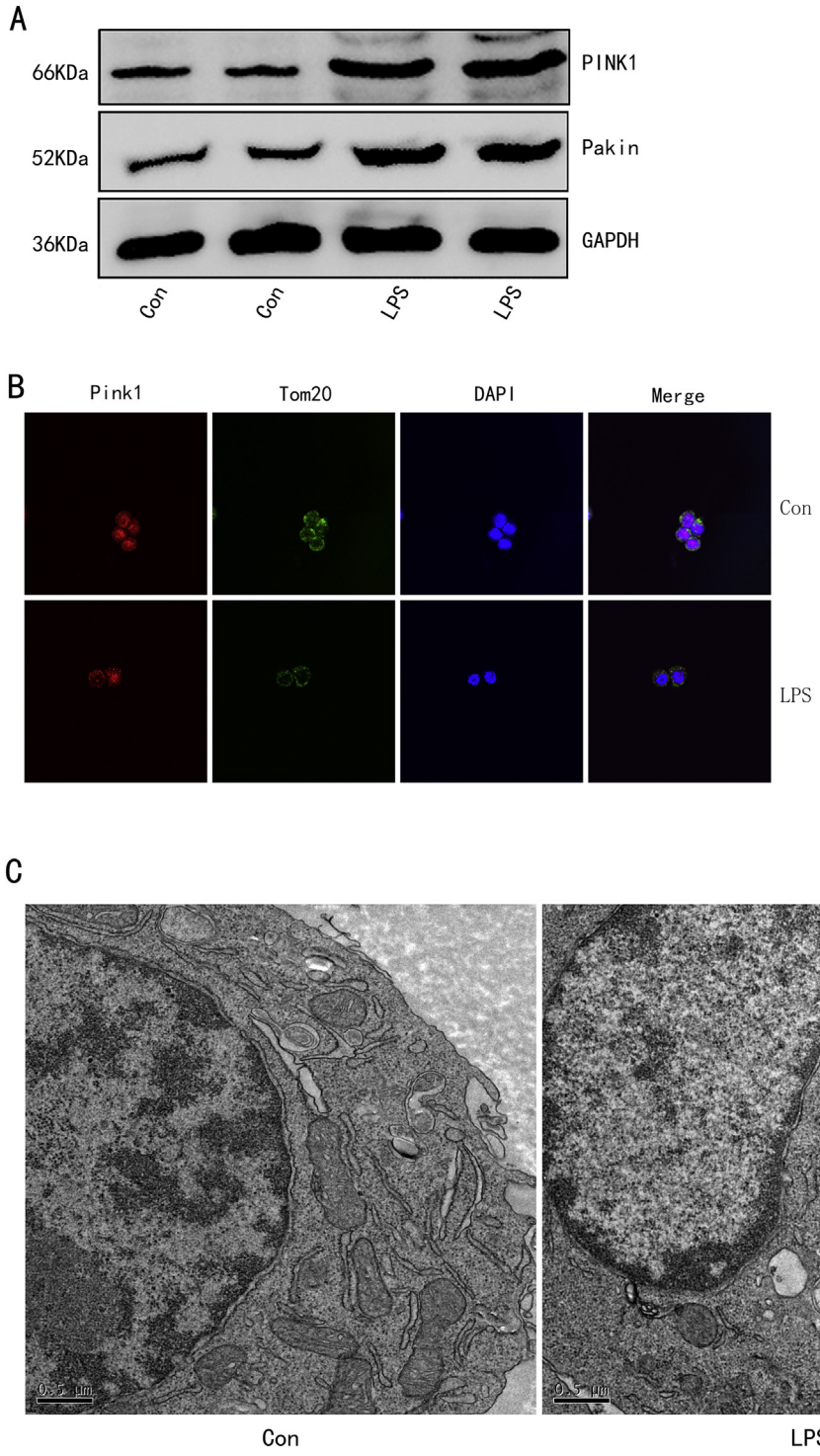

Fig. 3. PINK1-Parkin mediated mitophagy was induced in macrophages upon LPS stimulation. Raw 264.7 macrophages were stimulated without (con) or with LPS (1000 ng/ml) for 12 h. (A) The proteins of the PINK1-Parkin mitophagy pathway were analyzed by western blotting. (B) Cells were seeded in glass bottom cell culture dishes (1×10^5 cells/dish), then fixed and stained with antibodies and visualized by Olympus laser scanning confocal microscopy. (C) Cells were treated with or without LPS, then the cells were harvested and visualized by transmission electron microscope to assay the numbers of mitophagosomes and morphology. All data were expressed as the mean ± SD.

mitochondria. All of these data revealed that dexmedetomidine can promote autophagy/mitophagy, attenuate mitochondrial dysfunction and inhibit the LPS-induced mitochondrial-dependent apoptosis in macrophages.

4.6. Mitophagy is required for the protective effects of DEX on mitochondrial dysfunction and mitochondria-dependent apoptosis

With the previous data, we demonstrated that PINK1-mediated mitophagy played an important role during LPS treatment and that knockdown of PINK1 would lead to increased levels of inflammation, oxidative stress and apoptosis. However, there have been no lines of

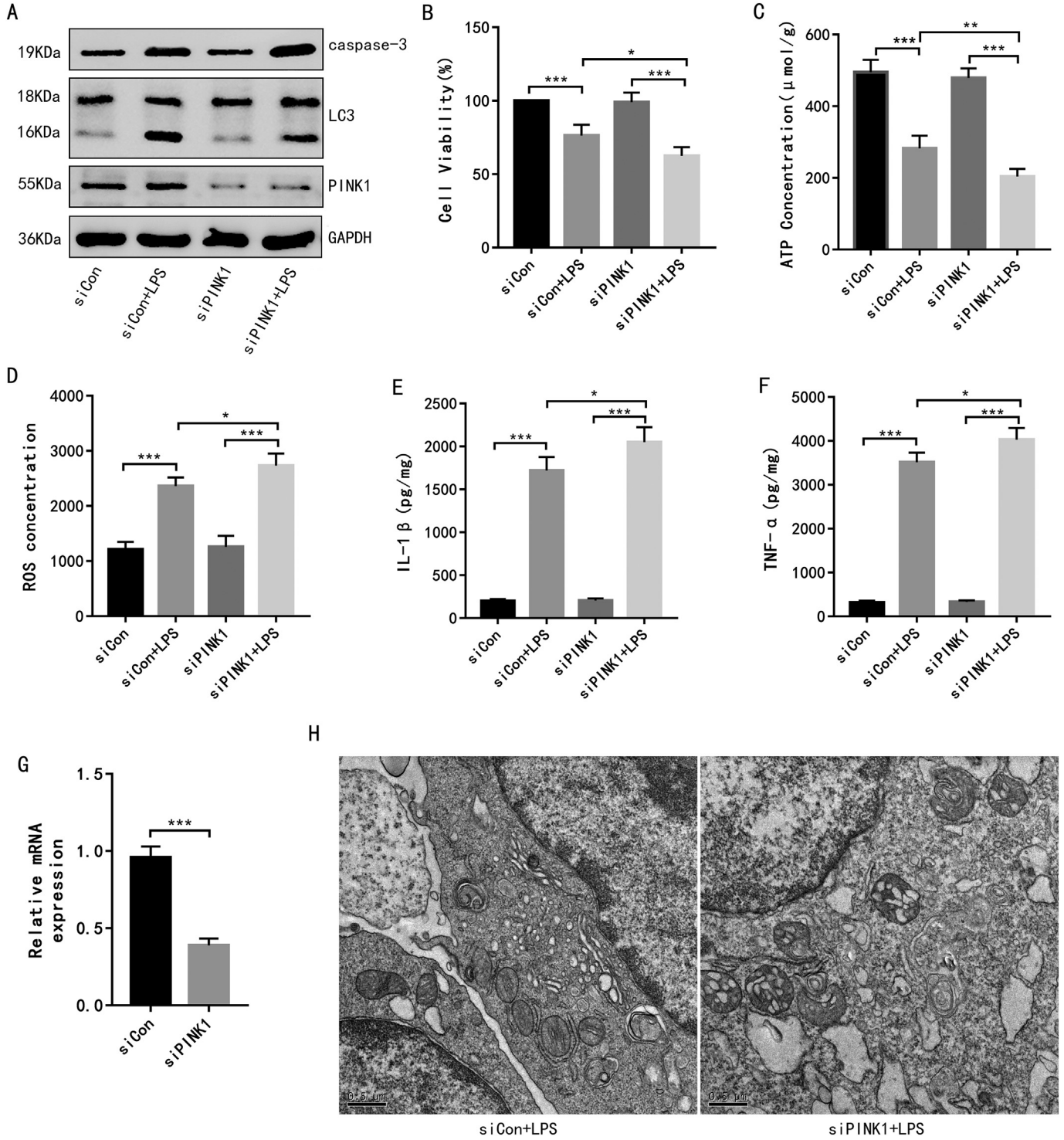

Fig. 4. Effects of PINK1 knockdown within macrophages against LPS-induced oxidative stress, autophagy changes, mitochondrial dysfunction and mitochondria-dependent apoptosis. Macrophages were transfected with control siRNA or siRNA targeting PINK1 for 24 h. The WT or transfected macrophages were then treated with or without LPS (1000 ng/ml) for 12 h. (A) The expression changes in caspase-3, LC3 and PINK1 were analyzed by western blotting. (B) Cell viability was measured by the CCK8 assay. (C) ATP concentration was measured by an ATP assay kit with a multimode reader. (D) ROS concentration was measured by an ROS assay kit with multimode reader. (E and F) IL-1β and TNF-α secretions were measured by enzyme-linked immunosorbent assay (ELISA). (G) Relative mRNA expression of PINK1 after transfection. (H) Cells were transfected for 24 h, treated with LPS for 12 h, and then the cells were harvested and visualized by transmission electron microscopy to assay the numbers of mitophagosomes and morphology. All data were expressed as the mean ± SD.

evidence about whether mitophagy is involved in the protective effect of DEX in this model. To test whether DEX exerts a protective effect via mitophagy, we used PINK1 siRNA to disrupt the process of mitophagy. The results showed that knockdown of PINK1 decreased ATP concentration (Fig. 6C), cell viability (Fig. 6B) and increased ROS production (Fig. 6D) upon DEX treatment in LPS-primed macrophages. Meanwhile, western blotting confirmed that knockdown of PINK1 expression led to more mitochondria-dependent apoptosis (Fig. 6A), and the anti-inflammatory effect was impaired as the secretions of IL-1β and TNF-α increased in the siPINK1 + LPS + DEX group when compared

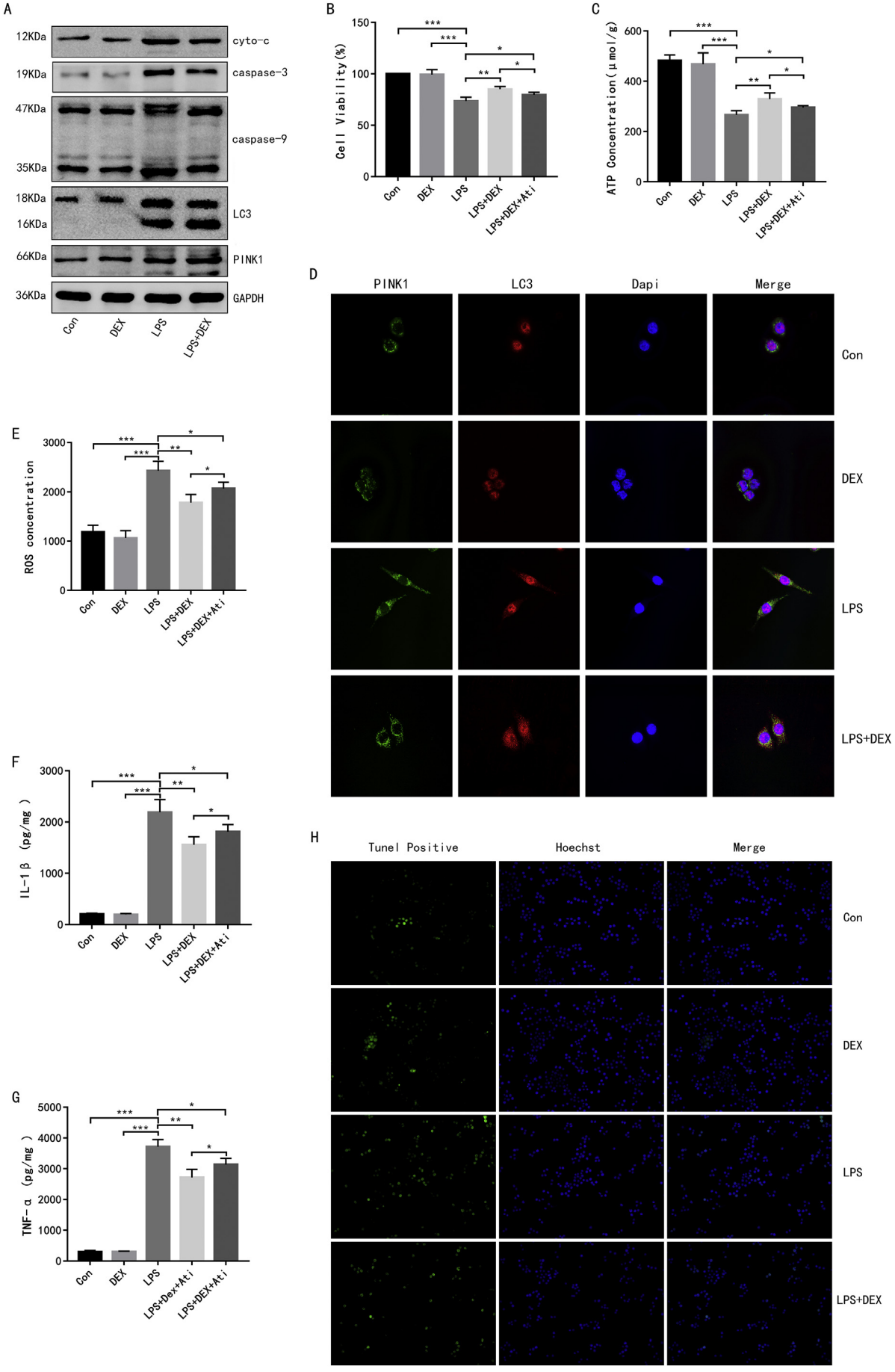

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Fig. 5. The effect of DEX on cell apoptosis, autophagy, mitochondrial function and inflammation. Raw 264.7 macrophages were stimulated without (con) or with LPS (1000 ng/ml) for 12 h, DEX (1 μM) was administered immediately after LPS administration, and ATI (atipamezole, 10 nM) was given after DEX. (A) The expressions of cyt-c, caspase-9, caspase-3, LC3 and PINK1 were measured by western blotting. (B) Cell viability was measured by CCK8 assay. (C and E) Mitochondrial function was assessed by ATP and ROS concentration via assay kits. (D) The expressions of LC3 and PINK1, and their colocalization in macrophages, were measured by immunofluorescence. (F and G) IL-1β and TNF-α secretions were measured by enzyme-linked immunosorbent assay (ELISA). (H) Apoptotic cells were measured by TUNEL assay. All data were expressed as the mean ± SD.

with the siCon + LPS + DEX group. From these data, we can summarize that PINK1-mediated mitophagy is required for the protective effects of DEX on mitochondrial dysfunction, inflammation and mitochondria-dependent apoptosis.

5. Discussion

Sepsis is a worldwide disease that has been described for almost 2000 years, but its clinical definitions have changed over time. The contemporary definition was achieved in the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3), in which sepsis was defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. Due to the high incidence, clinical cost and mortality of sepsis, every country has invested significant money into research funds and clinical assistance. With the efforts exerted over these years, the risk of death has declined; unfortunately, the number of people dying of sepsis continues to rise each year owing to the increased incidence of sepsis. In fact, sepsis has become the tenth leading cause of death in the USA, and the number of people dying of sepsis has reached nearly 200,000 annually, which is almost the same number of people dying from acute myocardial infarction and more than those who die from HIV, breast cancer or stroke. Although we have conducted much research on sepsis, many aspects remain unclear, and it is a considerable challenge. As important innate immune-reactive cells, macrophages are strategically located throughout the body tissues and can secrete pro-inflammatory and anti-inflammatory cytokines during the different stage of sepsis to regulate this process. Therefore, it is believed that macrophages play an important role in the development of infection in sepsis. In the late stages of sepsis, immune suppression, disorders and the apoptosis of the immune cells may act as the leading causes of death in patients with severe sepsis or septic shock [27,31].

LPS treatment is a widely used and well-accepted method to mimic inflammation for sepsis study in vitro and in vivo. DEX is a sedation drug that is reportedly protective against inflammation, apoptosis and attenuated organ dysfunction, especially in sepsis. Mitophagy, the specific autophagic elimination of dysfunctional or damaged mitochondria, is a quality control system. PINK1 is intimately involved with mitochondrial quality control by identifying damaged mitochondria and targeting specific mitochondria for degradation through PINK1/Parkin mediated mitophagy. In this study, we demonstrated that mitophagy played a protective role during LPS treatment and that PINK1 is needed for mitochondria clearance; meanwhile, we found that mitophagy is required for the protective effect of DEX. We used LPS treatment to developed the inflammation model successfully and found that mitophagy, inflammation and apoptosis are induced in RAW 264.7 cells during LPS treatment. Meanwhile, LPS treatment caused mitochondrial damage and mitochondria-dependent apoptosis, and knockdown of PINK1 further worsened the situation.

To test the effects of DEX on LPS-treated macrophages, we examined the concentration of inflammatory cytokines, including TNF-α (an inflammatory cytokine in the early stage) and IL-1β (an inflammatory cytokine which matured with damaged mitochondria). We then measured the levels of cellular ATP and MMP for mitochondrial function and the ROS concentration for oxidative stress, and we used CCK8, TUNEL assay and western blot assay to measure cell viability or apoptosis. The results showed that, compared with the LPS group, DEX treatment decreased the levels of inflammatory cytokines and ROS concentration, increased the cellular ATP, MMP and cell viability, and restrained cell apoptosis. However, the effect of DEX was impaired in PINK1 knockdown cells. These data demonstrate that the partial protective effect of DEX was mediated by PINK1. PINK1, the full name of which is PTEN-induced putative kinase 1, is a mitochondrial serine/threonine protein kinase encoded by the PINK1 gene. PINK1 is thought to protect cells from stress-induced mitochondrial dysfunction. The activation of PINK1 causes Parkin to bind to the outer membrane of depolarized mitochondria to induce mitophagy of those mitochondria. However, a healthy mitochondrion maintains a membrane potential that can import PINK1, where it is subsequently cleaved by PARL. Thomas G. and colleagues have reported that basal mitophagy occurs independently of PINK1 in mouse tissues of high metabolic demand, such as neural cells, pancreas and microglia. In that report, the authors found that basal mitophagy is comparable between PINK1 wild-type and knockout mice during normal circumstances. While under pathological conditions, the numbers of mitolysosomes show a significance difference between PINK1 wild-type and knockout MEFs [30]. Therefore, it is believed that PINK1-mediated mitophagy necessarily occurs when cells encounter various pathological situations.

It is known that various stressors induce the production of excessive ROS by the respiratory chain; furthermore, mitochondria are a prominent source of ROS and are subject to ROS-mediated injury. Mitochondrial dysfunction would lead to increased ROS production and aggravate mitochondrial damage, and in this case, ROS is a mitochondrial stressor that can lead to changes in mtDNA, mitochondrial transmembrane potential (ΔΨm), and mitochondrial lipids and proteins [32]. Loss of ΔΨm could be a critical trigger for PINK1 recruitment to damaged mitochondria [9], inducing mitophagy to clear dysfunctional/damaged mitochondria. In the intrinsic pathway leading to apoptotic death, changes in mitochondrial lipids and proteins result in the release of soluble mitochondrial intermembrane proteins, such as cytochrome *c*, into the cytosol. Upon release from the mitochondria, cytochrome *c* together with apoptotic protease-activating factor 1 (APAF1) and procaspase-9 form the apoptosome; subsequently, the active caspase-9 cleaves and activates caspase-3, and then all of these caspase activation events cause cell death via apoptosis [27]. Because dysfunctional mitochondria could cause apoptosis and be cleared by mitophagy, the relationship between mitochondria-dependent apoptosis and mitophagy is the primary interest of our research. The present study demonstrated that there is a close connection between dysfunctional mitochondria and apoptosis. Mitochondria are a dynamic organelle network, and dysfunctional mitochondria could be reshaped or recycled by fission and fusion or by mitophagy [9,33]. Mitochondrial fusion and division is an adaptive stress response that can dilute and segregate damaged mitochondria and form a continuously dynamic network within cells. However, during the maladaptive stress response, the damaged mitochondria will be recycled by mitophagy or lead to mitochondria-dependent cell apoptosis [34].

Some in vivo studies have found that DEX could ameliorate kidney ischemia-reperfusion injury and exert neuroprotective effects, as well as restrain the mortality rate and inflammatory responses to endotoxin-induced shock in rats, but the mechanism and cell signaling pathways are not clear. In this study, we focus on PINK1-mediate mitophagy and apoptosis. We demonstrate that mitophagy was induced in LPS-treated macrophages and that inhibiting mitophagy by PINK1 knockdown could increase the level of ROS and the expression of inflammatory cytokines and could aggravate mitochondrial dysfunction and

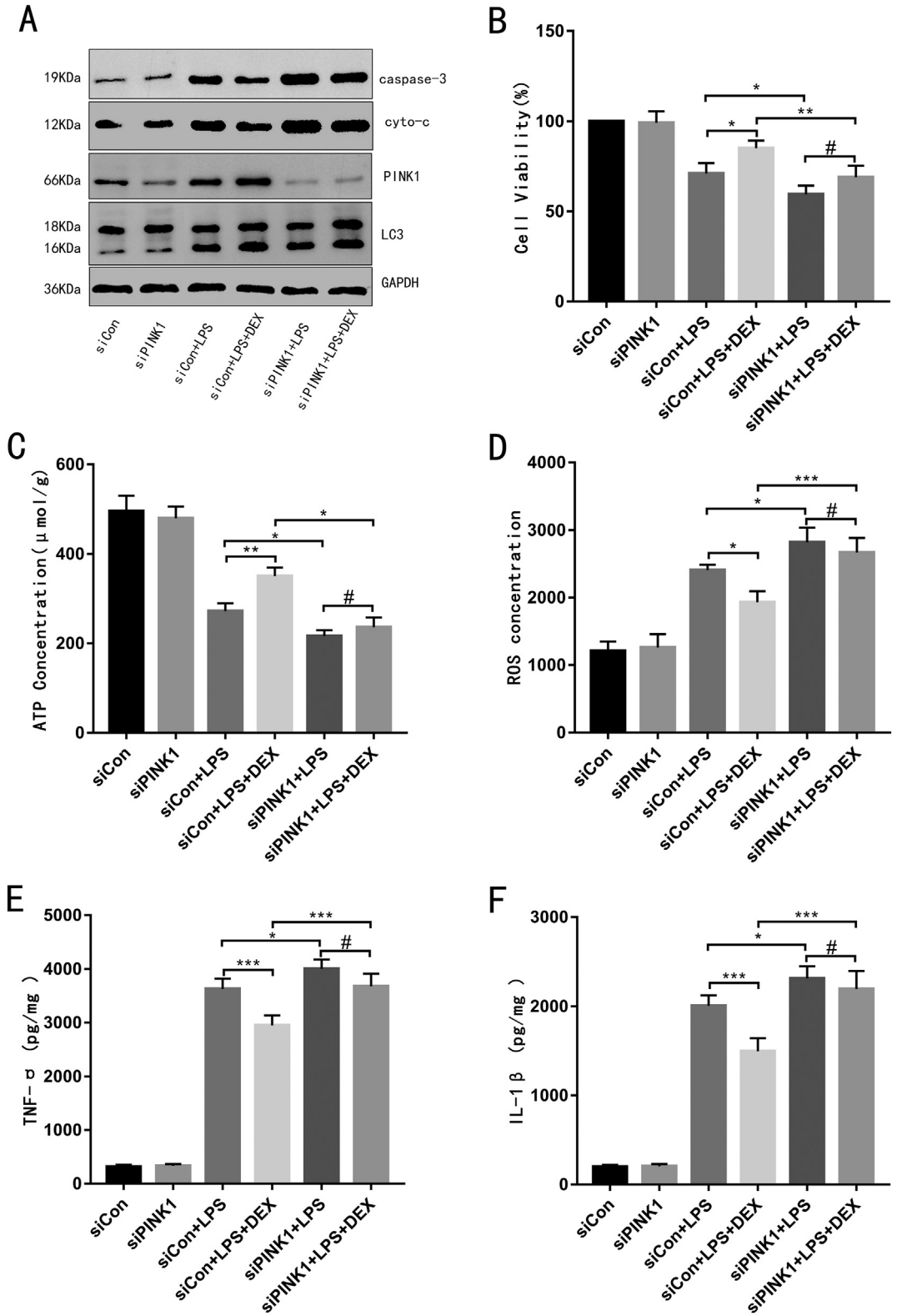

Fig. 6. The effect of PINK1 on DEX alleviates mitochondrial dysfunction and apoptosis and restrains inflammation. Macrophages were transfected with control siRNA or siRNA targeting PINK1 for 24 h, WT or transfected macrophages were all treated with LPS (1000 ng/ml) for 12 h, and then they were treated with or without DEX (1 μM) for 12 h. (A) The expressions of cyt-c, caspase-3, LC3 and PINK1 were measured by western blotting. (B) Cell viability was measured by CCK8 assay. (C and D) Mitochondrial function was assessed by ATP and ROS concentration via assay kits. (E and F) IL-1β and TNF-α secretion were measured by enzyme-linked immunosorbent assay (ELISA). All data were expressed as the mean ± SD.

apoptosis; these data indicate that mitophagy exerts a protective effect in LPS-treated macrophages. We next pondered whether mitophagy is involved in the protective effect of DEX. We used a nonspecific antagonist of α2 receptors, atipamezole, to block the activation of α2 receptors, but it failed to completely reverse the effect of DEX, which means that additional signaling pathways likely exist. We then found

that DEX could increase the expression of PINK1, promote the clearance of dysfunctional mitochondria by mitophagy and exert a protective effect. Meanwhile, PINK1 knockdown would impair the protective effect of DEX, and this indicates that the protective effect is mediated by PINK1.

In summary, our results demonstrated that PINK1-mediate mitophagy played a protective role in LPS-induced mitochondrial dysfunction and macrophage injury. Furthermore, the mechanism of the protective effect of DEX is correlated to the promotion of clearance of dysfunctional mitochondria via PINK1-mediated mitophagy. Mitochondria have emerged as central organelles that integrate energy metabolism, apoptosis and inflammatory responses. However, mitochondria are highly dynamic organelles and we focus only on PINK1-mediated mitophagy, inflammation and apoptosis; we must devote additional attention to other aspects of mitochondrial dynamics, and the mechanisms require more research to further validate the protective effects on cell apoptosis, inflammation and the promotion of cell survival. In vivo experiments are also required to elucidate the mechanisms and evaluate the therapeutic potential in inflammation models. Even though the anti-inflammation and anti-apoptosis effects remain unclear, these effects provide a promising anesthetic/sedative choice in treating sepsis patients.

Declaration of Competing Interest

The authors declare no relevant conflicts of interest.

Acknowledgments

This study was supported by grant from the National Natural Science Foundation of China (No. 81671888 to Yonghao Yu; 81601667 to Hongguang Chen), and Postgraduate Innovation Fund of 13th Five-Year comprehensive investment of Tianjin Medical University (YJSCX201720).

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