

Pink1/Parkin-mediated mitophagy regulated the apoptosis of dendritic cells in sepsis

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Abstract

Although the Pink1/Parkin-mediated mitophagy is closely related to inflammation and immunoregulation, its effect on apoptosis of splenic dendritic cells (DCs) in sepsis remains unknown. Here, our current study demonstrates that mitophagy is enhanced, and apoptosis of DCs is increased during sepsis in Wild Type (WT) mice. It also shows that mitophagy occurs in the process of the sepsis-induced apoptosis of DCs. Moreover, the level of mitophagy of Pink1-knockout (Pink1-KO) mice is lower than that of WT mice, while the apoptosis of DCs of Pink1-KO mice is increased further more than that of WT mice during sepsis. The mitochondrial dysfunction in DCs is aggravated in Pink1-KO mice during sepsis, which suggests that Pink1/Parkin-mediated mitophagy regulates the apoptosis of DCs by improving mitochondrial function. Thus, an impaired Pink1/Parkin-mediated mitophagy exacerbates the apoptosis of DCs and it might represent a novel therapeutic target to prevent sepsis in the future.

Introduction

Mitophagy, a selective autophagic process, is specifically targeted for eliminating dysfunctional or non-essential mitochondria from the population to maintain cellular homeostasis [1, 2]. There are two main mechanisms by which mitochondria are targeted for degradation at the autophagosome. One pathway is Pink1/Parkin-mediated mitophagy, and the other is receptor-mediated mitophagy [3]. Pink1 (PTEN-induced putative kinase protein1) and Parkin (E3 ubiquitin ligases)-mediated mitophagy response to a dissipation of the mitochondrial membrane potential (MMP) [4]. Mitochondrial function is markedly abnormal in sepsis, as evidenced by impaired adenosine triphosphate (ATP) synthesis and increased reactive oxygen species (ROS) production, which is also a key factor in the development of apoptosis and multiple organ dysfunction syndrome (MODS) [5, 6]. Our previous study found that MMP reduction and mitophagy were induced by the late septic inflammatory factor high mobility group box-1 protein (HMGB1), which is closely related to the expression of the Parkin mitochondrial receptor protein Mfn2, suggesting that mitophagy may be involved in the pathogenesis of sepsis [7, 8]. Mitophagy is prevalent in eukaryotic cells. It has been reported that mitophagy is vitally important for the proper differentiation of T lymphocytes. Inhibition of mitophagy leads to an increase in the number of mitochondria and ROS content in mature T lymphocytes, resulting in impaired peripheral survival [3, 9]. Another recent report highlights the critical role of mitophagy in the maintenance of innate immune homeostasis [10]. Dendritic cells (DCs), as a bridge between innate and adaptive immunity, are the key antigen presenting cells (APC) that activate T lymphocytes in the body. Moreover, the number and function of DCs are closely related to the regulation of the immune system and the stability of the internal environment [11]. DCs apoptosis is a critical link in the regulation of the balance between tolerance and immunity through multiple pathways, and its abnormal occurrence can be a trigger for autoimmunity [12]. Although DCs apoptosis can lead to the development of immunosuppressive states and organ failure during sepsis, its mechanism still needs further clarity [11, 13–15]. Therefore, we hypothesized that during sepsis, mitophagy was engaged in the regulation of the apoptosis of DCs by a mechanism that may involve the Pink1-Parkin signaling pathway. In the current study, we attempted to analyze the mechanism of the Pink1-Parkin signaling pathway in

regulating the apoptosis of DCs in sepsis and to further elucidate the essential link between mitophagy and DCs apoptosis. We showed that mitophagy is upregulated and the level of DCs' apoptosis is increased during sepsis. Furthermore, the level of mitophagy was significantly reduced in Pink1-KO mice compared to WT mice at 24 h after CLP. Silencing the Pink1 gene resulted in a further increase in the apoptosis of DCs. Therefore, it was suggested that blocking the Pink1-Parkin signaling pathway by knockout of Pink1 gene could effectively inhibit mitophagy and further aggravate the apoptosis of DCs. These findings expanded the understanding of Pink1/Parkin-mediated mitophagy regulating apoptosis in immune cells during sepsis, which may represent a novel therapeutic target in sepsis-related apoptosis of DCs.

Materials And Methods

1.Reagents and Abs

RPMI 1640 were purchased from TDBscience (Tianjin, China). Fetal bovine serum (FBS) was from Gibco-Invitrogen Corporation (Garlsbad, CA, USA). EasySep Mouse CD11c Pos Slctn Kit was obtained from STEMCELL (Vancouver, Canada). Anti-LC3B antibody, anti-Parkin antibody, anti-Cleaved Caspase-3 antibody, anti-Bcl-2 antibody and anti-Bax antibody were purchased from CST (Boston, USA). Anti-PINK1 antibody, anti-P62/SQSTM1 antibody, anti-Tom20 antibody, anti-ATP5A1 antibody and anti-UQCRC1 antibody were from Proteintech (Chicago, USA). Anti- β -actin antibody, anti-rabbit IgG secondary antibody, and anti-mouse IgG secondary antibody was obtained from Applygen (Beijing, China). Mouse lymphocyte separation medium was purchased from TBDscience (Tianjin, China). Anti-mice CD11c MircoBeads and MS columns were from Miltenyi Biotech (Bergisch Gladbach, Germany). Annexin V-PE apoptosis detection kit was obtained from BD Biosciences (Franklin Lakes, NJ, USA). One step TUNEL apoptosis assay kit, Fluo-4 AM (calcium ion fluorescent probe, 2mM) and reactive oxygen species assay kit were purchased from Beyotime (Shanghai, China). Dylight649 AffiniPure rabbit anti-goat IgG (H + L) was from Abbkine (California, USA). MitiBright Green and Mitophagy Detection Kit were obtained from Dojindo (Kyushu, Japan). DAPI solution was from Solarbio (Beijing, China).

2.Mice and Experimental Groups

Male wild type (WT) C57BL/6 mice (6 to 8 weeks, 18–22 g) were purchased from the Laboratory Animal Institute, Chinese Academy of Medical Sciences (Beijing, China). Pink1-KO mice in the C57BL/6 background were obtained from Nanjing Biomedical Research Institute of Nanjing University. All mice were housed on a 12-h light-dark cycle with controlled temperature (21°C to 23°C) and provided with a standard rodent diet and water *ad libitum* throughout all experiments. All studies were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all methods were authorized by the ethics committee of the Laboratory Animal Ethics Committee of Wenzhou Medical University. Male wild type C57BL/6 mice were randomly divided into the CLP 6 h, CLP 12 h, CLP 24 h, CLP 48 h, CLP 72 h, and Sham groups. Pink1-KO mice and WT mice were randomly divided into the WT, WT + CLP 24 h, Pink1-KO, Pink1-KO + CLP 24 h groups.

3. Cecal ligation and puncture (CLP)

Experimental sepsis model can be established by CLP because of its ease of reproducibility and resemblance to human sepsis progression [16]. Sepsis was induced in male C57BL/6 mice (6- to 8-weeks old, 18 to 22g weight) by CLP as previously described [17]. In brief, mice were anesthetized with chloral hydrate (500mg/kg) by intraperitoneal perfusion. The abdominal skin of mice was disinfected with mice immobilized. An approximately 1 cm middle abdominal incision was made and the cecum was exposed and ligated with 4 – 0 silk suture without causing intestinal obstruction. Then the cecum was completely punctured once with a 22-gauge needle and was returned to the peritoneal cavity. The abdomen was closed in 2 layers with 4 – 0 silk suture and mice were then injected with 1 ml sterile saline to resuscitate and prevent dehydration. Sham-operated controls underwent laparotomy and bowel manipulation without cecal ligation and puncture.

4. Cell Preparation

C57BL/6J mice of the CLP 6 h, CLP 12 h, CLP 24 h, CLP 48 h and CLP 72 h groups (each group: n = 3) were killed at 6 h, 12 h, 24 h, 48 h and 72 h post-CLP separately. Mice of the WT + CLP 24 h group (n = 3) and Pink1-KO + CLP 24 h group (n = 3) were killed at 24 h post-CLP. Mice of the Sham group (n = 3), the WT group (n = 3) and the Pink-KO group (n = 3) were sacrificed at 24 h post-sham operation. All mice in these groups were sacrificed by cervical dislocation to remove the spleens. Cells were dispersed through a 30 μ m stainless steel mesh and collected after centrifugation at 1500 rpm for 5 min, and they were resuspended in PBS. Cells were added rat serum and set for 5 min. Component A and Component B were mixed in equal volumes of 25 μ l/ml and added to cells to incubate for 5min. Using RapidSpheres to shake thoroughly, DCs were separated by passing the cell suspension over a magnetic-activated cell sorter MS column held in EasySep magnetic separator (Vancouver, Canada). The DCs were then resuspended and counted.

5. Polymerase Chain Reaction (PCR)

The gene expression of Pink1-KO mice was confirmed by polymerase chain reaction. Spleens were harvested to isolated total DNA by using a rapid animal genomic DNA isolation kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. The following sequences were used: Wild Type, 5'-ACT GCC ACA CTC AGT CCT TG-3' (forward), 5'-GCA CTA CAG CGA ACT GCA TC-3' (reverse), producing a 373 bp fragment; and Mutant, 5'-GCA CTA CAG CGA ACT GCA TC-3' (forward), 5'-GCC AGA GGC CAC TTG TGT AG-3' (reverse), producing a ~ 500 bp fragment.

6. Western blotting analysis

Cells were treated as described previously and harvested by cold Radioimmunoprecipitation Assay (RIPA) lysis buffer containing protease inhibitors and phosphatase inhibitors. After centrifugation at 12,000g for 20 min at 4°C, the protein concentration was assayed using the Bicinchoninic acid (BCA) assay. Equal amounts of protein (30 μ g) were separated by 10–12% sodium dodecyl sulfate (SDS)- Polyacrylamide gel

electrophoresis (PAGE) and transferred onto 0.45- μ m polyvinylidene fluoride (PVDF) membranes (Millipore, CA, USA). After the membranes were blocked with 5% fat-free dry milk in Tris-buffered saline containing Tween-20 (TBST), they were incubated with anti-Tom20, anti-Pink1, anti-Parkin, anti-LC3, anti-p62, anti-Bcl-2, anti-Bax, anti-cleaved-caspase-3, anti-ATP5A1 and anti-UQCRC1 at 4°C overnight. Membranes were washed three times and incubated with the HRP- conjugated secondary antibodies. Protein bands were visualized by using ECL reagents and performed with the ChemiDic™ XRS + Imaging System (Hercules, CA, USA). Densitometry analysis of each band was quantified using Image Lab software and normalized against those of β -actin protein in each sample.

7. Flow cytometric analysis

Annexin V-FITC and PI staining: Cells were stained with PE Annexin V and 7-ADD. DCs were collected and washed twice with cold PBS, and then resuspended in 200 μ l 1 \times binding buffer, to which were added 2.5 μ l PE Annexin V and 2.5 μ l 7-ADD. After incubation for 15 minutes in darkness at room temperature (RT), the cells were analyzed by flow cytometry.

Intracellular calcium measurement: We measured the density of intracellular calcium in DCs with flow cytometry using the Ca²⁺-sensitive fluorescent dye Fluo-4/AM. Cells were incubated for 30 minutes at 37°C. Cells were washed by centrifugation and they were incubated for another 30 minutes at 37°C. The intracellular calcium was analyzed by flow cytometry.

8. TUNEL staining

DCs were fixed with 4% paraformaldehyde for 30min. And cells were centrifuged with PBS at 400 rpm for 5min and were broken the membrane with 0.3% Triton X-100 for 5 min at room temperature. Cells were centrifuged with PBS at 400 rpm for 5min and incubated at 37 °C for 1 h with 50 μ l TUNEL detection solution. Cells were centrifuged with PBS at 400 rpm for 5min, and 100 μ l liquid was left on the slide. 10 μ l DAPI was added to stain the nucleus. Cells were stored at -20°C and were used to detect apoptosis by fluorescent microscope.

9. Microscopy

Cells were collected and added an appropriate amount of DCFH-DA (10 μ mol/L) and they were incubated for 30 minutes at 37°C in the dark. Cells were washed and centrifuged using RPMI1640 and 100 μ l of the base solution was reserved for making slides. The level of ROS in cells was determined with the fluorescent microscope (Leica, Germany) according to manufacturer's instructions.

Cells were collected and the membrane was ruptured on Digitonin for 2 min on ice fixed with 4% paraformaldehyde (PFA) for 1 h. Cells were blocked with 1% BSA for 1 h. They were then incubated with LC3B primary antibody (1: 200) at 4°C overnight. After 3 PBS washes, cells were incubated with fluorescent secondary antibody (1:500) at room temperature for 1 h. Cells were washed with RPMI1640 and were incubated with MitoBright probe for 10 min at room temperature. Finally, cells were washed with

RPMI1640 3 times, leaving about 100µl of the substrate drop on the slide. The nucleus was stained with 10µl DAPI solution and the solution was placed in a wet box at -20°C. The localization of LC3 on mitochondria was observed by confocal microscopy (Leica, Germany). In addition, cells were collected and incubated using Mtpagy Dye at 37°C for 30 minutes and centrifuged with RPMI1640. Then cells were washed. The nucleus was stained with 10µl DAPI solution and the solution was stored in a humidified box at -20°C. The level of mitophagy was observed by confocal microscopy (Leica, Germany).

10. Transmission electron microscopy

After treatment, cells in Eppendorf were prefixed in 2.5% glutaraldehyde in PBS for one hour at room temperature, then stored at 4°C overnight before processing. Thin sections on grids were observed in a transmission electron microscope. Images were acquired with a charge-coupled device camera (AMT).

11. Statistical analysis

All results are presented as the mean \pm standard deviation (SD). Data analyses were carried out using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). Statistical significance between different groups was assessed by one-way ANOVA. Comparisons between two groups were made using Student's *t* test using SPSS 25.0 software. $P < 0.05$ was considered statistically significant.

Results

1. The apoptosis of DCs was induced during sepsis.

CD11c⁺ DCs were obtained with MicroBeads. We chose two times, 24 h and 72 h after CLP, to examine the apoptosis of DCs in mice spleen based on the above experiments. To verify the sepsis-induced apoptosis of DCs, the abundance of apoptosis-associated proteins was monitored at 24 h, 72 h after CLP, and sham group (Fig. 1A). The Bcl-2/Bax ratio was decreased in the splenic DCs of mice after CLP compared with those in sham group ($P < 0.05$). And the Bcl-2/Bax ratio was lower at 72 h after CLP compared with 24 h after CLP ($P < 0.05$). The level of cleaved-caspase-3 protein was also higher in the 24 h group than in the sham group ($P < 0.05$), whereas no significant difference in 72 h group and the sham group was observed. In particular, the results of flow cytometry (Fig. 1B) and TUNEL (Fig. 1C) showed that the apoptosis rate of DCs at 24 h after CLP was more than 50% higher than that of the sham group. Furthermore, even though the apoptosis rate of DCs at 72 h after CLP was higher than that of the sham group, the trend was different from that of the 24 h group: there was no significant difference between the flow cytometry results, the TUNEL result was lower, and the WB result was higher.

2. Pink1/Parkin-mediated mitophagy was induced during sepsis.

To investigate the effect of Pink1/Parkin signaling pathway on mitophagy during sepsis, the abundance of mitophagy-associated proteins was monitored at 6 h, 12 h, 24 h, 48 h, 72 h after CLP, and the sham group. The protein abundance of Tom20, Pink1, and Parkin were all upregulated in the splenic DCs of

mice after CLP compared with those in the sham group (Fig. 2). It showed that Pink1 tended to combine with the TOM complex rather than degrade, leading to enhanced Parkin activation, which was an essential pathway for Pink1/Parkin to activate mitophagy. Overall, the evidence indicated that sepsis could induce Pink1/Parkin-mediated mitophagy in DCs.

Meanwhile, our observation by confocal microscopy also showed that the expression level of LC3 in the splenic DCs of mice at 24 h after CLP was significantly increased compared with the sham group, and there was co-localization of LC3 with mitochondria (Fig. 3A). Mitophagy specific fluorescence staining showed that mitophagy was significantly enhanced in the splenic DCs of mice at 24 h after CLP (Fig. 3B). In addition, the swelling of mitochondria, the disorder of cristae arrangement and the fusion with autophagosomes in the splenic DCs mitochondria were observed by transmission electron microscopy at 24 h after CLP (Fig. 3C).

3. The mitophagy of DCs was effectively attenuated by blocking the Pink1/Parkin signaling pathway.

Using PCR to observe the expression of Pink1 gene, the results showed that mutant products were appeared in all Pink1-KO mice DNA (Fig. 4A). It was also shown that Pink1 protein was not expressed in the splenic DCs using Western Blot (Fig. 4B). To investigate the difference of mitophagy between WT mice and Pink1-KO mice during sepsis, the LC3 expression and mitophagy were observed by confocal microscopy. It showed that blocking the Pink1/Parkin signaling pathway resulted in a remarkable reduction of LC3 expression in DCs (Fig. 4C, D). Moreover, the abundance of mitophagy-associated proteins was monitored at 24 h after CLP (Fig. 5). At 24 h after CLP, the abundance of Tom20 protein in the DCs of WT mice was markedly higher than that of the sham group, while the abundance of Tom20 protein in the DCs of Pink1-KO mice was significantly lower than that of WT mice ($P < 0.05$) (Fig. 5A). At 24 h after CLP, the abundance of Parkin protein in the DCs of WT mice was markedly high, while the abundance of Parkin protein in the DCs of Pink1-KO mice was significantly lower than that of WT mice ($P < 0.05$) (Fig. 5B). At 24 h after CLP, the abundance of LC3 protein and p62 protein was significantly increased in DCs of WT mice, whereas the expression levels of these two proteins were decreased in Pink1-KO mice ($P < 0.05$) (Fig. 5C).

4. Pink1/Parkin-mediated mitophagy had anti-apoptotic effects during sepsis.

We speculated that Pink1/Parkin-mediated mitophagy played a vital regulatory role in sepsis-induced apoptosis of DCs. Thus, we used flow cytometry, TUNEL, and Western Blot to examine the effect of knockdown of the Pink1 gene on the apoptosis of DCs after CLP. Western Blot was used to examine apoptosis-related proteins at 24 h after CLP (Fig. 6A), and it showed that the Bcl-2/Bax ratio was significantly low in WT + CLP group, and the Bcl-2/Bax ratio was further reduced in Pink1-KO mice compared with WT + CLP 24 h ($P < 0.05$). The abundance of cleaved-caspase-3 protein in DCs of Pink1-KO mice was significantly lower than that in WT + CLP group ($P < 0.05$). Both flow cytometry (Fig. 6B) and TUNEL (Fig. 6C) results showed an increase in splenic DCs apoptosis rate at 24 h after CLP, and the increase in DCs apoptosis rate was more pronounced in Pink1-KO mice than in the WT + CLP 24 h group ($P < 0.05$).

5. Pink1/Parkin-mediated mitophagy may contribute to anti-apoptotic effects by improving mitochondria function.

We hypothesized that Pink1/Parkin-mediated mitophagy might contribute to the anti-apoptotic effect by improving mitochondrial function. We examined the effects of Pink1/Parkin-mediated mitophagy on DCs mitochondrial function in sepsis in terms of cellular ATP synthesis, Ca^{2+} levels, and ROS content. At 24 h after CLP, the abundance of ATP5A1 protein in DCs of WT mice was significantly lower than those in the sham group, whereas the abundance of ATP5A1 protein in DCs of Pink1-KO mice was lower than those in the WT + CLP 24 h group ($P < 0.05$). In addition, CLP induced a decrease in UQCRC1 protein abundance in DCs, whereas Pink1-KO mice had a further decrease in UQCRC1 protein abundance in DCs compared with WT + CLP 24 h group ($P < 0.05$) (Fig. 7A). The changes of Ca^{2+} in DCs were observed by flow cytometry with Fluo-4 AM calcium fluorescent probe incubation, and the result showed that Ca^{2+} in DCs increased significantly at 24 h after CLP ($P < 0.05$) (Fig. 7B). However, there was no significant difference between Pink1-KO + CLP group and WT + CLP group. After DCFH-DA incubation, the changes of ROS in DCs were observed by fluorescence microscope. The result showed that at 24 h after CLP, the ROS content in DCs of WT mice was markedly increased, and the content of ROS in DCs of Pink1-KO mice increased compared with WT + CLP group ($P < 0.05$) (Fig. 7C).

Discussion

Sepsis, defined as life-threatening organ dysfunction caused by a dysregulated host response to infection, is a major public health concern [18]. A period of continuous immunosuppressive state leads to alterations in both the innate and adaptive immune response during sepsis [19], but the mechanisms are largely elusive. Sepsis-induced immune cell apoptosis is considered to be a key factor in the immunosuppression of sepsis. Apoptosis of lymphocytes, including innate immune cells and adaptive immune cells, is closely related to a higher risk of secondary infections and poor outcomes [20]. Being key antigen-presenting cells, DCs are closely related to the occurrence and development of sepsis [13]. It has been shown that the number of DCs in the spleen and mesentery of CLP model decreased significantly at 12 h [21]. The DCs apoptosis not only induces the self-growth and maturation disorders but also causes the differentiation of naïve T cells into Treg [22]. The response incompetence of T cells is caused by the change of expression levels of DCs function-related surface molecules (MHC, CD40, CD80 and CD86). Moreover, an immune tolerance state is induced in sepsis due to the abnormal secretion of cytokines by DCs [14].

Recent studies have shown that the change of DCs immune function in sepsis is involved with various mechanisms, such as apoptosis, activation of Wnt signaling pathway, Toll-like receptor (TLR)-dependent signaling, and ROS generation [11]. Herein, we demonstrated that the apoptosis rate in DCs was significantly higher than that in the sham group using flow cytometry and TUNEL assay. Therefore, inhibition of DCs apoptosis may be a novel target for the treatment of sepsis.

Depolarization of the MMP due to increased oxidative stress in sepsis is a strong signal to trigger mitophagy [23, 24]. Mitophagy is caused by a decrease in MMP that activates the Pink1-Parkin signaling pathway to ubiquitinate autophagy receptors [25, 26]. Our study also demonstrated the increased mitophagy at 24 h after CLP surgery in DCs. It has been reported that Pink1/Parkin-mediated mitophagy is enhanced by cardiac overexpression of Beclin-1 in response to LPS stimulation, which in turn reduces cardiac inflammation and improves cardiac function [27]. It has been found that Pink1-KO mice have significantly increased mortality compared to WT mice after CLP procedure, whereas HMGB1 and NLRP3 inhibitors are able to suppress the septic inflammatory response and organ dysfunction in Pink1-KO mice, which indicates the organ-protective effect of Pink1 in the course of sepsis [28].

With the exception of their roles as antigen presenting cells, DCs produce a multitude of cytokines that stimulates cells of the innate and adaptive immune system [29]. Thus, we speculated that mitophagy was potentially involved in the alteration of the DCs function and quantity in the immunosuppressive septic state. Our study compared changes in splenic DCs mitophagy in WT mice and Pink1-KO mice under normal physiological conditions and 24 h after CLP. We provided evidence that DCs mitophagy was markedly reduced in Pink1-KO mice compared with WT mice at 24 h after CLP, which indicated DCs mitophagy might be effectively inhibited by the interruption of Pink1/Parkin signaling pathway.

It has been cleared that DCs apoptosis is one of the main mechanisms responsible for the large reduction of DCs in sepsis [30]. Recent literature suggests that mitochondrial injury, apoptosis, and Pink1-mediated mitophagy can be induced by LPS-stimulated macrophages. Meanwhile, the Pink1-mediated mitophagy plays a protective role in LPS-induced inflammation and apoptosis by phagocytosis of dysfunctional mitochondria [31]. It has been observed that Akt1 kinase-mediated mitophagy has an anti-apoptotic effect in alveolar macrophages [32]. In studies of other diseases, Pink1 has also been found to play a vital role in the inhibition of cellular apoptosis [33–35]. Thus, we hypothesized that sepsis-induced DCs apoptosis was regulated by Pink1/Parkin-mediated mitophagy. Our study showed that blocking the Pink1/Parkin signaling pathway led to a further increase in DCs apoptosis, which proved our hypothesis was correct.

Mitochondrial dysfunction in sepsis is mainly manifested as ATP depletion due to inhibition of oxidative phosphorylation (OXPHOS), excessive ROS production, the disorder of Ca²⁺ homeostasis, and the release of pro-apoptotic proteins [36]. There has increasing evidence that mitochondria themselves have become major executors of apoptosis and that their dysfunction can trigger apoptosis and at last contribute to organ failure [37, 38].

Oxidative stress injury, caused by ROS excess, can be alleviated with mitophagy by specifically removing damaged mitochondria [24]. In a recent study, it has been reported that knockdown of the Pink1 gene was found to result in mitochondrial ROS generation, NLRP3 inflammasome activation, and increased renal impairment [33, 39]. Moreover, the decreased level of Parkin in idiopathic pulmonary fibrosis (IPF) lung fibroblasts contributed to the production of mitochondrial ROS [35]. To date, a significant decrease in the function of the electron transfer chain (ETC) is observed in the striatum of Pink1-KO mice (40). Moreover,

it has been shown that the mitochondrial Ca^{2+} level can be increased by the mutation of Pink1 gene [41, 42]. The evidence above has demonstrated that the Pink1-Parkin signaling pathway is involved in the regulation of ROS, ETC, and Ca^{2+} . Furthermore, mitochondrial damage and increased inflammasome hyperactivation and mortality are associated with the inhibition of mitophagy by knocking down the SESN2 (Sestrin2) gene in mice during sepsis [43], which suggests that mitophagy has anti-mitochondrial damage and anti-inflammatory effects in sepsis. Therefore, we speculated that Pink1/Parkin-mediated mitophagy might play its anti-apoptotic effect by improving mitochondrial function. We examined the pathophysiological mechanism in terms of cellular ATP synthesis, Ca^{2+} level, and ROS content, which confirmed that DCs mitochondrial dysfunction could be delayed by the Pink1/Parkin-mediated mitophagy during sepsis.

However, it is likely that Pink1/Parkin-mediated mitophagy is just one of the mechanisms by which mitophagy attenuates DCs apoptosis induced by sepsis. An in-depth study of signaling pathways and regulatory mechanisms by which mitophagy regulates DCs and even other immune cell apoptosis may provide a novel target for effective intervention in the immunosuppressive state of sepsis.

Declarations

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Conflicts of interest: Not applicable

Availability of data and material: All data generated or analyzed during this study are included in this published article.

Code availability: Not applicable

Authors' contributions: Yaolu Zhang and Yinan Luo contributed equally to this work. Yinan Luo, Yongming Yao, and Zhongqiu Lu designed the research. Yaolu Zhang, Linan Luo, Zhong Xiao, Kang Wang, and Xinyong Liu performed the research. Yaolu Zhang, Yinan Luo, Zhong Xiao, Kang Wang, Xinyong Liu, Longwang Chen and Guangju Zhao analyzed the data. Longwang Chen and Guangju Zhao contributed reagents. Yinan Luo, Yaolu Zhang, Yongming Yao, and Zhongqiu Lu wrote the paper.

Ethics approval: All studies were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all methods were authorized by the ethics committee of the Laboratory Animal Ethics Committee of Wenzhou Medical University.

Consent to participate: Written informed consent for participation was obtained from all participants.

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Figures

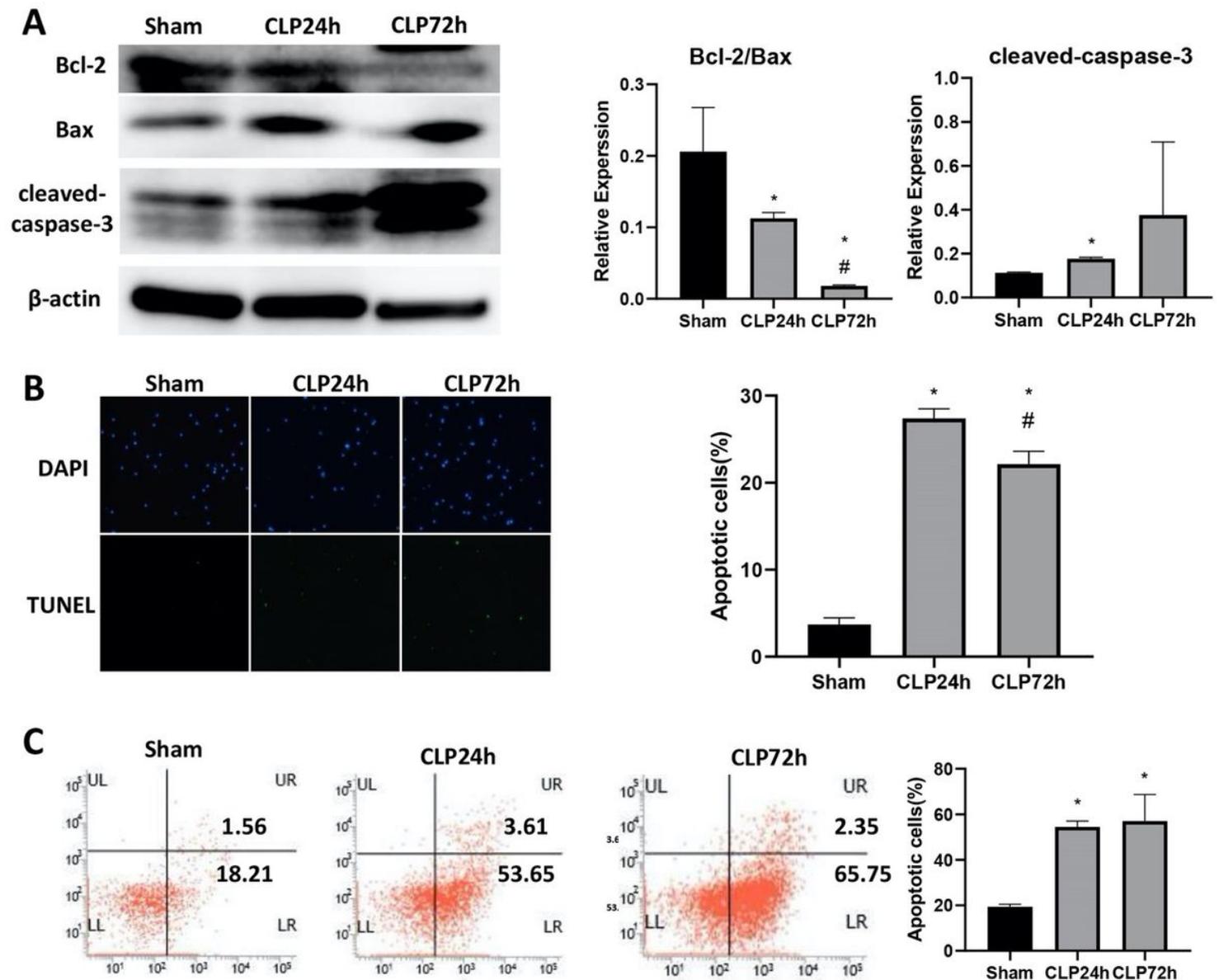


Figure 1

(A) Western blot of the expression level of the apoptosis-related protein Bcl-2, Bax and cleaved-caspase-3 among different groups (the Sham, CLP 24 h and CLP 72 h groups) in splenic DCs of mice (n=3) (* P<0.05 versus the Sham group; # P<0.05 versus the CLP 24 h group). (B) TUNEL of the change of the apoptotic rates among different groups (the Sham, CLP 24 h and CLP 72 h groups) in splenic DCs of mice (TUNEL, n=3) (* P<0.05 versus the Sham group; # P<0.05 versus the CLP 24 h group). (C) Flow cytometry of the change of the apoptotic rates among different groups (the Sham, CLP 24 h and CLP 72 h groups) in splenic DCs of mice (FCM, n=3) (* P<0.05 versus the Sham group).

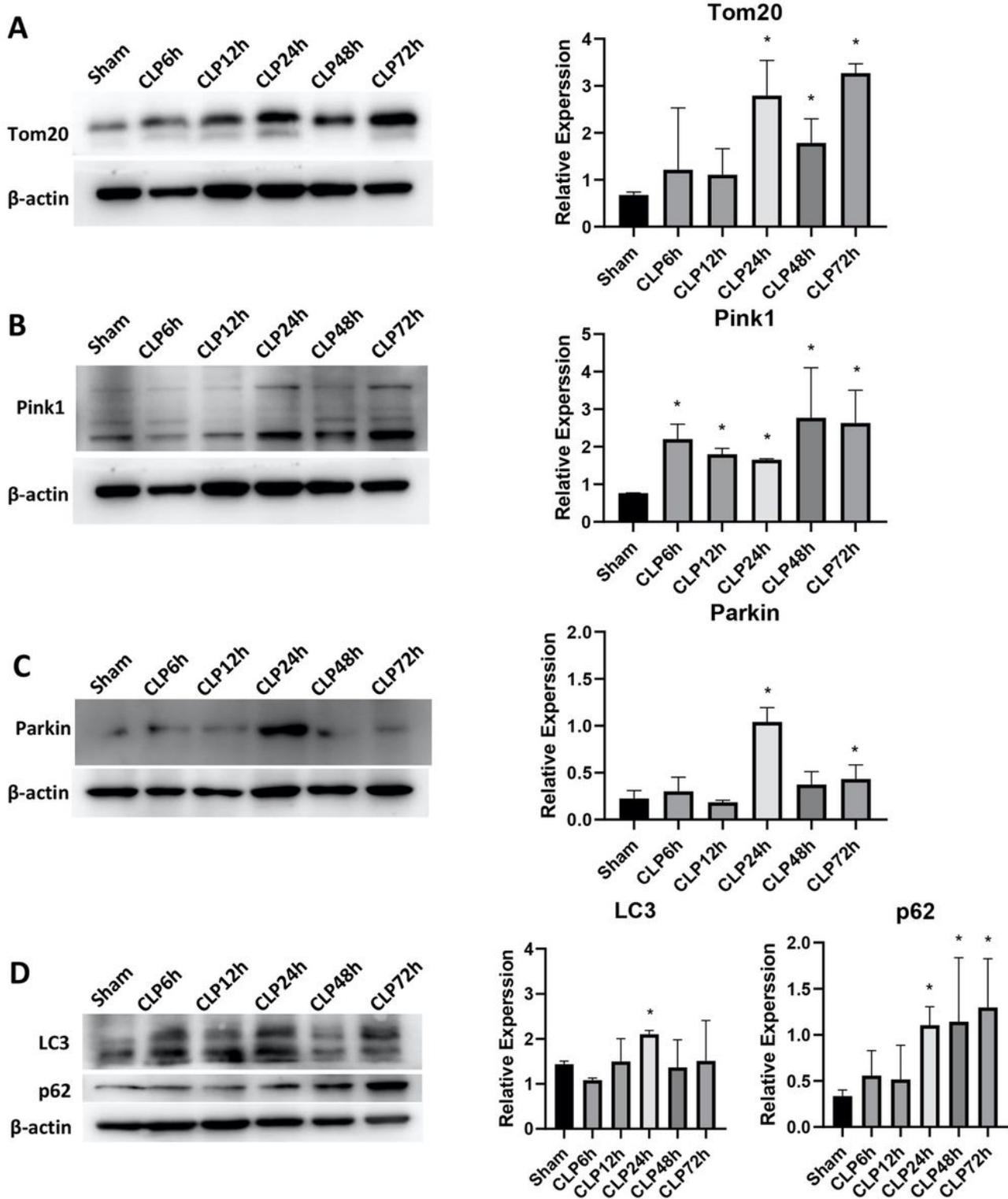


Figure 2

Western blot of the expression level of the mitophagy-related protein Tom20, Pink1, Parkin, LC3 and p62 among different groups (Sham, CLP 6 h, CLP 12 h, CLP 24 h, CLP 48 h and CLP 72 h groups) in splenic DCs of mice (n=3) (* P<0.05 versus the Sham group).

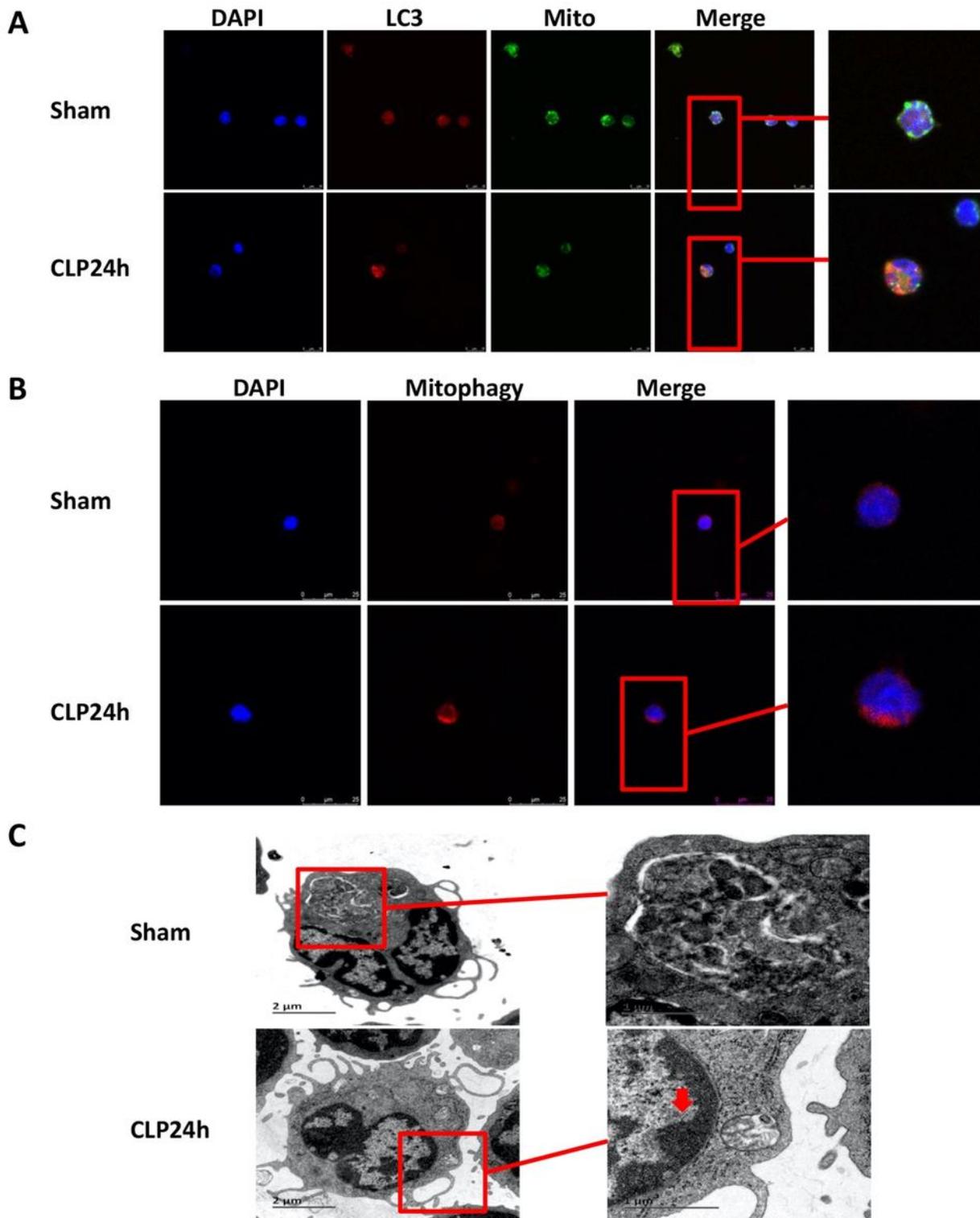


Figure 3

(A) TUNEL of the expression of LC3 in splenic DCs and its localization on mitochondria of mice in the Sham and the CLP 24 h groups (confocal microscopy $\times 1200$). (B) TUNEL of the effect on mitophagy after CLP for 24 h in splenic DCs of mice in the Sham and the CLP 24 h groups (confocal microscopy $\times 1200$). (C) Transmission electron microscope of the effect on mitophagy after CLP for 24 h in splenic DCs of mice in the Sham and the CLP 24 h groups (transmission electron microscope $\times 20000$, $\times 50000$).

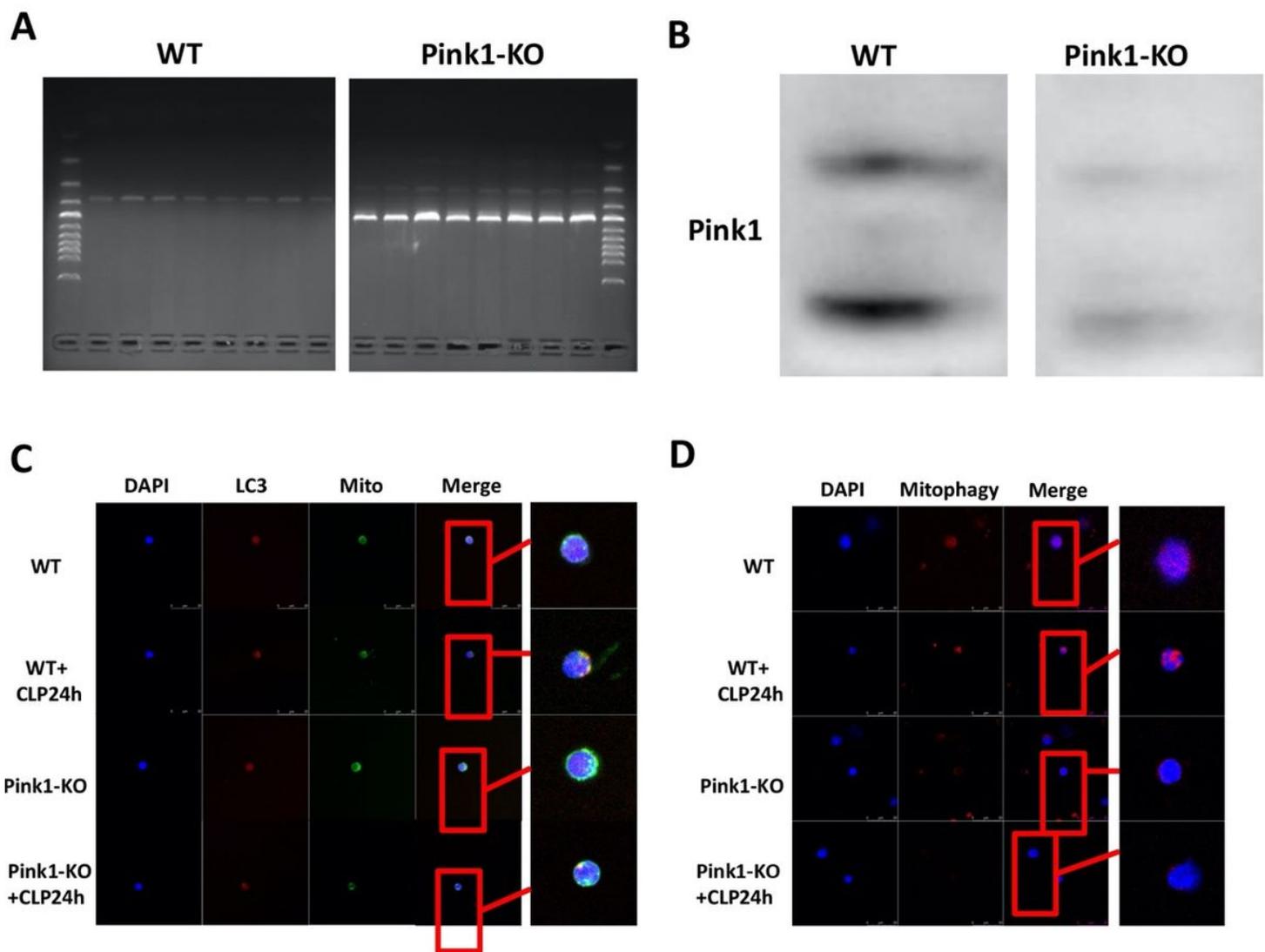


Figure 4

(A) PCR of the identification of Pink1 gene in WT mice and Pink1-KO mice. Wild type=373 bp; Mutant= \sim 500 bp. (B) Western blot of the expression level of Pink1 in splenic DCs of WT mice and Pink1-KO mice. (C, D) Confocal microscopy of the effect to LC3, mitochondria and mitophagy of DCs among different groups (WT, WT+CLP 24 h, Pink1-KO and Pink1-KO+CLP 24 h) of mice. (C) The localization and expression of LC3 in splenic DCs and its mitochondria; (D) The change of mitophagy after CLP for 24 h in splenic DCs.

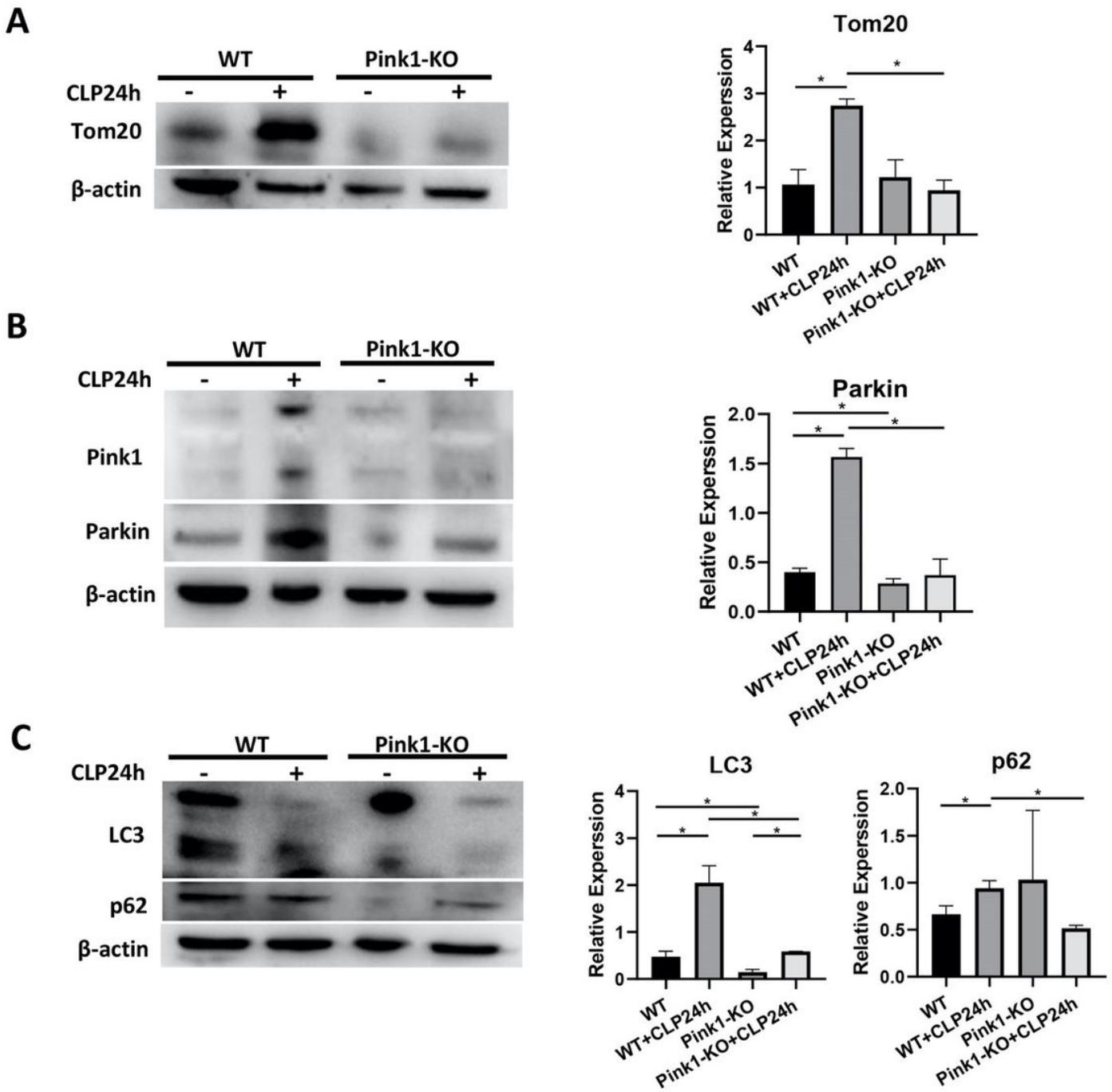


Figure 5

Western blot of the expression level of the mitophagy-related protein Tom20, Pink1, Parkin, LC3 and p62 among different groups (WT, WT+CLP 24 h, Pink1-KO and Pink1-KO+CLP 24 h) in splenic DCs of mice (n=3) (* P<0.05).

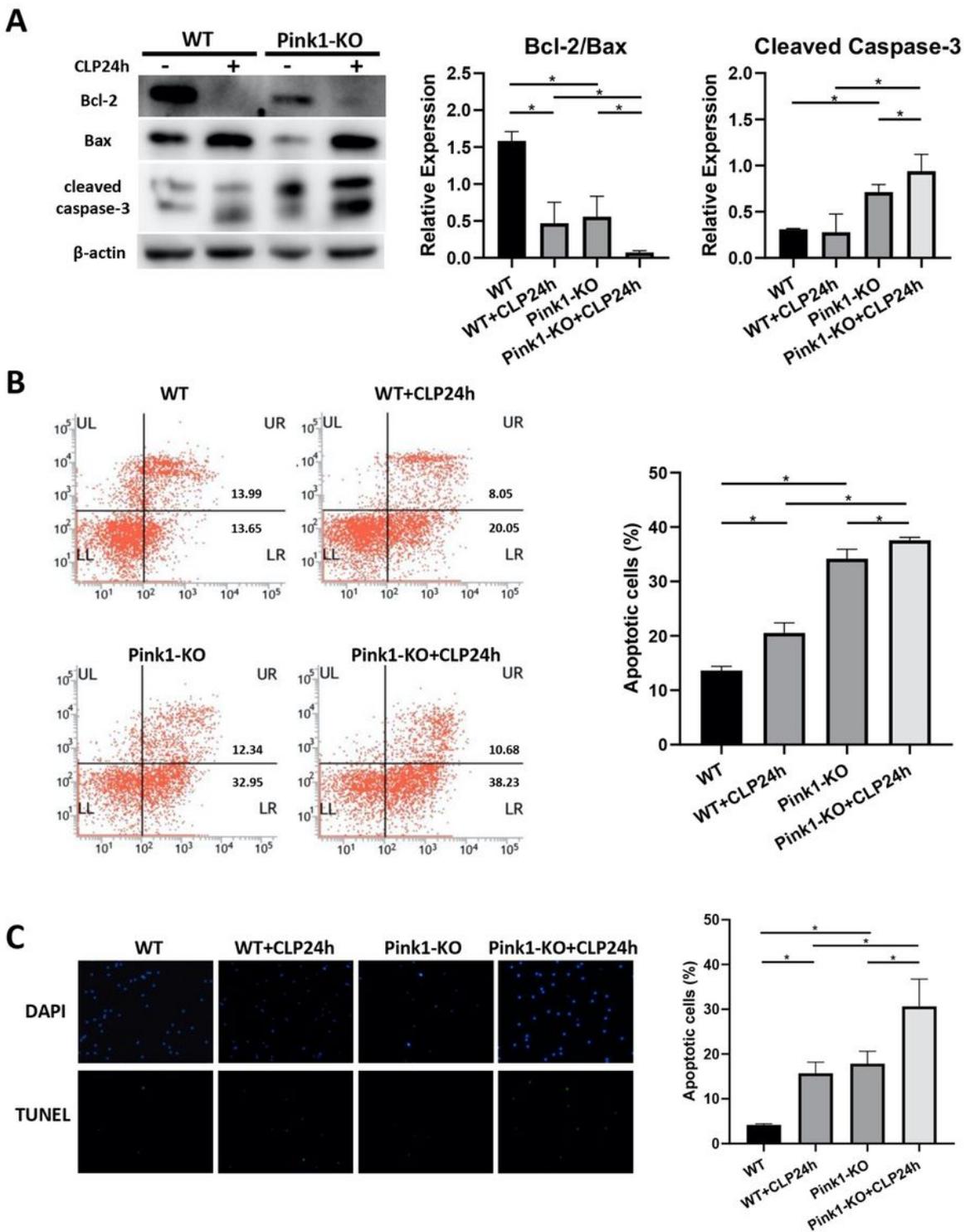


Figure 6

(A) Western blot of the expression level of the apoptosis-related protein Bcl-2, Bax and cleaved Caspase-3 among different groups (WT, WT+CLP 24 h, Pink1-KO and Pink1-KO+CLP 24 h) in splenic DCs of mice ($n=3$) ($* P < 0.05$). (B) Flow cytometry of the change of the apoptotic rates of DCs among different groups (WT, WT+CLP 24 h, Pink1-KO and Pink1-KO+CLP 24 h) of mice (FCM, $n=3$) ($* P < 0.05$). (C) TUNEL of the

change of the apoptotic rates of DCs among different groups (WT, WT+CLP 24 h, Pink1-KO and Pink1-KO+CLP 24 h) of mice (TUNEL, n=3) (* P<0.05).

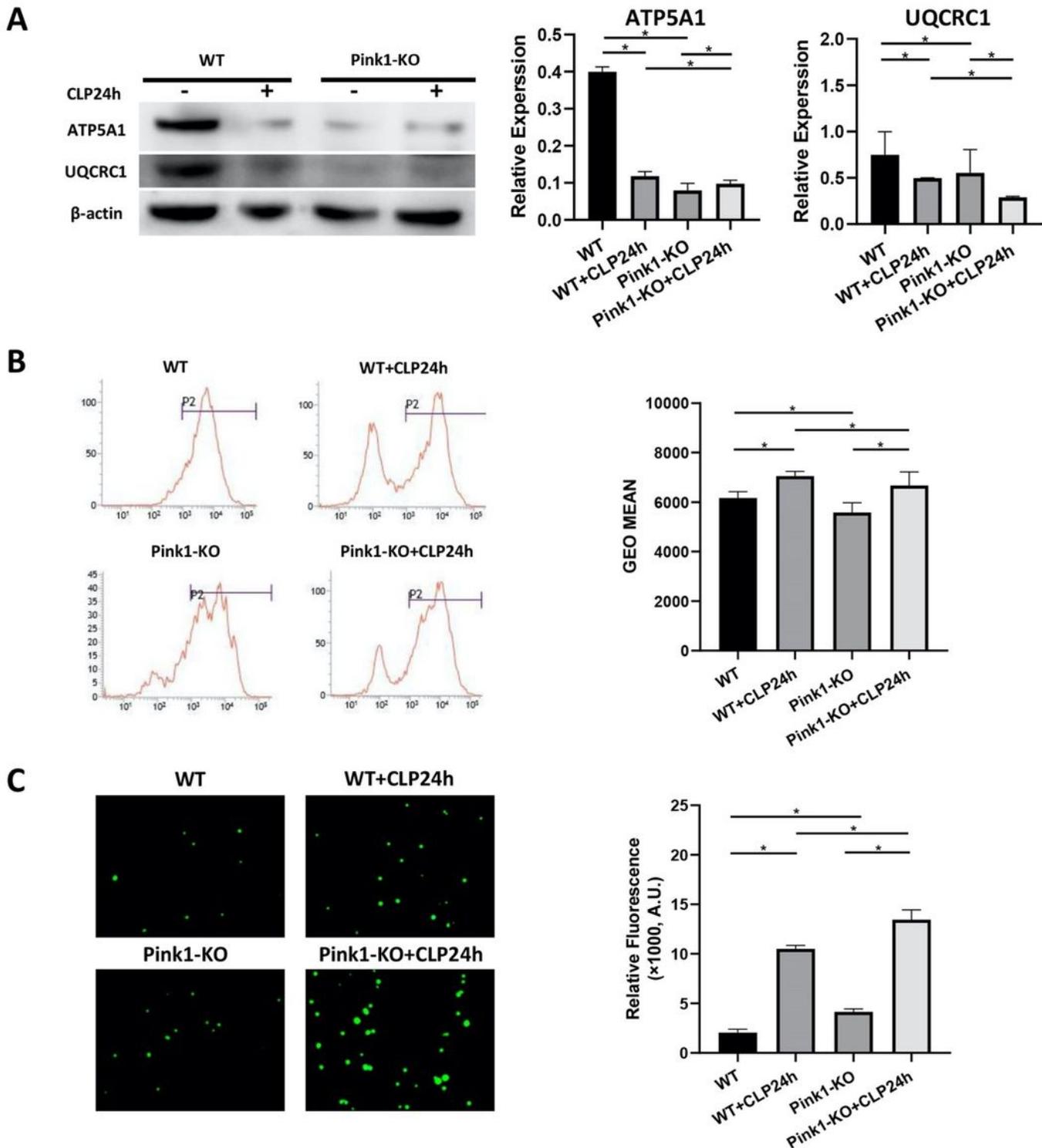


Figure 7

(A) Western blot of the expression level of the respiratory chain-related protein ATP5A1 and UQCRC1 in DCs among different groups (WT, WT+CLP 24 h, Pink1-KO and Pink1-KO+CLP 24 h) of mice (n=3) (* P<0.05). (B) Flow cytometry of the effect to the Ca²⁺ in DCs among different groups (WT, WT+CLP 24 h,

Pink1-KO and Pink1-KO+CLP 24 h) of mice (FCM, n=3) (* P<0.05). (C) Fluorescent microscope of the effect to the ROS in DCs among different groups (WT, WT+CLP 24 h, Pink1-KO and Pink1-KO+CLP 24 h) of mice (fluorescent microscope, n=3) (* P<0.05).