Materials and methods

Animal ethics statement

All animal experiments of the C57BL/6 mice were approved by the Institutional Animal Ethics Committee of Jiangsu Ocean University, and animal care was in accordance with the institutional guidelines.

Strains, reagents and antibodies

Pseudomonas aeruginosa (*P. aeruginosa*, ATCC 15468) is kept in our laboratory. SYBR Green qPCR Mix (MQ10101) was purchased from Monad. Polyclonal antibodies of HSP60 (15282-1-AP), TIM23 (11123-1-AP), GAPDH (10494-AP) and HRP-conjugated affinipure goat anti-rabbit IgG (H+L) (SA00001-2) were all purchased from Proteintech. LC3B (T55992) was from Abmart. FITC goat anti-rabbit IgG (E031220) was from Earthox. Genomic DNA extraction kit (DP304), Mitotracker red CMXRos (C1049B), Cell mitochondria isolation kit (C3601), Mitochondrial membrane potential assay kit with JC-1 (C2006), Reactive oxygen species (ROS) assay kit (S0033S), Cell counting kit-8 (CCK8, C0037), and LDH cytotoxicity assay kit (C0016) were purchased from Beyotime. The ATP content assay kit (BL852A) was purchased from Biosharp. ELISA kits of mouse IL-17 (BMS6001), IFN-γ (88-7314-88), TNF-α (88-7324-22) were purchased from Invitrogen. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP, C2759) and Mdivi-1 (M0199) used in the in *vitro* experiments were purchased from Sigma. *N*-acetyl-L-cysteine (NAC, S1623) was provided by SELLECK. CCCP (HY-100941) used for in *vivo* experiments was purchased from MCE. Mdivi-1 (M303472) used for in *vivo* experiments was sourced from Aladdin.

Preparation of *P. aeruginosa* suspension

P. aeruginosa was cultured in LB medium at 37°C for 18-24 h. When the optical density at 600 nm (OD_{600}) reached to 0.6-0.8, *P. aeruginosa* at the logarithmic growth stage was centrifuged at 8,000 × g for 5 min. For *in vitro* assays, *P. aeruginosa* was resuspended in 1640 cell culturing medium and counted under microscope. For *in vivo* assays, *P. aeruginosa* was resuscitated with 1 mL sterile normal saline. OD value of the suspended bacterial solution was re-analyzed and convert into CFU/mL (an OD_{600} of 0.5 corresponds to 10^9 CFU/mL(Li et al., 2021). The *P. aeruginosa* suspension was diluted to 10^9 CFU/mL for mouse assays.

Establishment of a *P. aeruginosa* infected mouse peritoneal macrophage model

The primary mouse peritoneal macrophages were prepared and the *P. aeruginosa* infection

model was established referring to our previously published article (Zhang et al., 2021). Specially, the multiplicities of infection (MOIs) of *P. aeruginosa* and macrophages were set at 1, 5, 10, respectively. The incubation time was 6 h. The treatment concentrations of CCCP (an mitophagy agonist), Mdivi-1 (an mitophagy inhibitor), and NAC (an ROS scavenger) were 10 μmol/L, 20 μmol/L, 2 mmol/L, respectively.

Transmission electron microscopy (TEM) observation

P. aeruginosa-stimulated or unstimulated cells were collected, fixed in 4% tissue cell fixative, negatively stained with tungsten phosphate and observed for mitochondrial morphological changes by TEM.

Immunofluorescence

The immunofluorescence assays were conducted referring to our previously published article (Zhang, et al., 2021). Specially, the mitochondria was stained with MitoTracker. The dilution times for LC3B and FITC goat anti-rabbit IgG were both 1:200.

Real time fluorescence quantitative PCR (qPCR) detection of mitochondrial DNA (mtDNA) contents

P. aeruginosa genomic DNA was extracted using a genomic DNA extraction kit. qPCR target gene primer sequence information is: mouse mtATP6-forward: 5′-CAGTCCCCTCCCTAGGACTT-3′, mouse mtATP6-reverse: 5′-TCAGAGCATTGGCCATAGAA-3′. qPCR housekeeping gene primer sequence information is: mouse Rpl13a-forward:5′-GGGCAGGTTCTGGTATTGGAT-3′, mouse Rpl13a-reverse:5′-GGCTCGGAAATGGTAGGGG -3′ (Zhang et al., 2019). The qPCR reaction mixture was prepared using *P. aeruginosa* genomic DNA as a template, mtATP6 and Rpl13a primer sets as targeted genes. The reaction program was set referring to the instructions of SYBR Green qPCR mix. The mtDNA fold changes were calculated using the $2^{-\Delta\Delta CT}$ method.

Western blotting (WB) detection of mitochondrial molecular chaperone heat shock protein 60 (HSP60) and endosomal protein T cell immunoglobulin mucin family 23 (TIM23)

The WB assays were carried out referring to our previously published article (Zhang, et al., 2021). Specially, the housekeeping protein was GAPDH. The dilution times of primary antibodies were 1:5000 for HSP60, 1:1000 for TIM23 and 1:5000 for GAPDH. The dilution time of secondary antibody was 1:5000.

Mitochondrial membrane potential changes

The absorbance value of mitochondrial aggregates in purified *P. aeruginosa*-stimulated or unstimulated cells were detected at $OD_{500\ nm}$ using a full-wavelength microplate system according to the instructions of mitochondrial membrane potential assay kit with JC-1.

ATP content assay

The intracellular ATP content of *P. aeruginosa* stimulated or unstimulated cells was determined by referring to the instructions of the ATP content assay kit, and the OD_{340nm} wavelength was measured using a UV spectrophotometer.

ROS release assay

The ROS release levels in *P. aeruginosa*-stimulated or unstimulated cells were measured by referring to the instructions of the ROS assay kit, and the levels of fluorescent signal release at 488 nm were observed using a laser confocal microscope (Olympus, Japan).

Determination of cytokine secretion levels

The levels of cytokine release from *P. aeruginosa*-stimulated or unstimulated cell culture supernatants were determined by referring to the instructions of mouse IL-17, IFN-γ, TNF-α ELISA kit.

CCK8 assay for cell viability

The cell viability was determined in *P. aeruginosa*-stimulated or unstimulated cells using a full-wavelength zymography at OD_{450} nm by referring to the CCK8 assay kit instructions.

LDH release level determination

Referring to the instructions of the LDH assay kit, LDH release levels were measured in *P. aeruginosa*-stimulated or unstimulated cells using a full-wavelength microplate system at OD_{490} nm.

Establishment of a mouse acute *P. aeruginosa* pulmonary infection model

BALB/C male mice were selected, 6-8 weeks old, weighing 18-25 g. Mice were divided into six groups, namely the negative control group, *P. aeruginosa* infection group, CCCP group, Mdivi-1 group, CCCP + *P. aeruginosa* group and Mdivi-1 + *P. aeruginosa* group, with 5 mice in each group. During the first 5 days, CCCP or Mdivi-1 was administered intraperitoneally at doses of 5 mg /kg body weight (BW)/ day and 20 mg /kg BW/ d. On the 6th day, mice were infected with *P. aeruginosa* suspension by noninvasive intratracheal instillation (200 μL/mouse) (Revelli et al., 2012). Mice in the negative control group, CCCP group and Mdivi-1 group were injected with equal

dose of sterile saline. The survival rate of mice in each group was observed and recorded at 0, 3, 6, 12, 24 h, respectively (0 h was calculated when all mice recovered their mobility after endotracheal injection).

Hematoxylin-eosin (H&E) staining

Fresh lungs were dissected and fixed with 4% tissue fixation solution. Paraffin embedded sections were stained with hematoxylin and eosin. 200 × imaging images were taken with an optical microscope (OLYMPUS, Japan).

Statistical analysis

GraphPad Prism 8 software was used to produce histograms of targeted genes or protein fold changes and the results were labeled as mean ± standard deviation (SD) in the graphs. ImageJ software was used to perform grayscale value calculations on images from immunoblotting experiments. One-way analysis of variance (ANOVA) followed by *t*-test was conducted to make statistical analysis. p values < 0.05 were considered statistically significant, while results without statistical significance of $p > 0.05$ are expressed as ns.

References

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