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# Staphylococcus aureus induces mitophagy via the HDAC11/IL10 pathway to sustain intracellular survival

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## Abstract

**Background** The immune evasion and prolonged survival of Staphylococcus aureus (*S. aureus*) within macrophages are key factors contributing to the difficulty in curing osteomyelitis. Although macrophages play a vital role as innate immune cells, the mechanisms by which *S. aureus* survives within them and suppresses host immune functions remain incompletely understood.

**Methods** This study employed confocal microscopy, flow cytometry, ELISA, and siRNA technology to assess the survival capacity of *S. aureus* within macrophages and the impact of inflammatory cytokines on its persistence. Proteomics was used to investigate the potential mechanisms and differential proteins involved in *S. aureus* intracellular survival. Additionally, confocal microscopy, flow cytometry, Mdivi-1 intervention, and Western blot were utilized to validate the role of mitophagy in supporting *S. aureus* survival. The study further explored how the HDAC11/IL10 axis enhances mitophagy to promote intracellular *S. aureus* survival by using HDAC11 overexpression, siRNA, and rapamycin intervention combined with confocal microscopy and flow cytometry.

**Results** The findings demonstrated that IL10 promotes mitophagy to clear mitochondrial reactive oxygen species (mtROS), thereby enhancing the intracellular survival of *S. aureus* within macrophages. Additionally, we discovered that the transcriptional repressor of IL10, HDAC11, was significantly downregulated during *S. aureus* infection. Overexpression of HDAC11 and the use of the autophagy activator rapamycin further validated that the HDAC11/IL10 axis regulates mitophagy via the mTOR pathway, which is essential for supporting *S. aureus* intracellular survival.

**Conclusion** This study reveals that *S. aureus* enhances IL10 production by inhibiting HDAC11, thereby promoting mitophagy and mtROS clearance, which supports its survival within macrophages. These findings offer new insights

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into the intracellular survival mechanisms of *S. aureus* and provide potential therapeutic approaches for the clinical management of osteomyelitis.

**Keywords** Histone deacetylase 11, Intracellular survival, Interleukin 10, Mitophagy, Macrophage, Mitochondrial reactive oxygen species, Staphylococcus aureus

## Introduction

Osteomyelitis, a common catastrophic orthopedic complication, arises from microbial infections, resulting in inflammation of bones, joints, and marrow [1]. Staphylococcus aureus (*S. aureus*) is a principal pathogen responsible for osteomyelitis, comprising approximately 30% of all cases [2]. The WHO has classified it as one of the antibiotic-resistant bacteria severely threatening human health, associated with high mortality rates globally [3]. Osteomyelitis due to *S. aureus* is highly prevalent, significantly harmful, and poses a substantial burden. In patients with prosthetic joint infections, the rate of reinfection post-revision surgery is as high as 30%, with *S. aureus* responsible for more than 50% of these recurrences [4, 5]. Therefore, exploring the pathogenic mechanisms and reasons for recurrence of osteomyelitis caused by *S. aureus* holds substantial clinical importance.

Studies show that after being phagocytized by phagocytes, *S. aureus* can evade intracellular immune responses and utilize the cells as a "Trojan horse," persisting within neutrophils and macrophages for hours to weeks and facilitating secondary transmission and recurrent infections [6]. Our earlier studies revealed that post-local vancomycin treatment, *S. aureus* continues to persist intracellularly in periprosthetic joint infection patients, with macrophages identified as the primary host cell type for colonization [7]. Therefore, elucidating the specific mechanisms behind the persistent survival of *S. aureus* within macrophages is crucial for effectively treating bone infections caused by this pathogen.

Macrophages are key immune cells in the body's defense against pathogenic microbes, capable of recognizing and engulfing bacteria, and eliminating intracellular pathogens through the release of pro-inflammatory factors, ROS, and antimicrobial peptides [8]. Mitochondrial Reactive Oxygen Species (mtROS), predominantly generated during mitochondrial oxidative phosphorylation, play a vital role in the immune response of professional phagocytes against intracellular bacteria [9]. Once Toll-like receptors (TLRs) detect bacteria, mitochondria are recruited to phagosomes, leading to an increase in mtROS production [10]. On one hand, mtROS can compromise bacterial cell membrane integrity, irreversibly damage DNA, and inhibit bacterial proliferation [11]. On the other hand, mtROS activate nuclear factor kappa-B (NF-kB), leading to the production of pro-inflammatory factors and the inflammasome, and triggering "ROS-induced ROS release" (RIRR) [12]. This causes irreversible mitochondrial damage and leads to cell apoptosis or necrosis, exposing intracellular bacteria to extracellular antibiotics and bactericidal agents, thereby disrupting their intracellular niche [13]. Under physiological conditions, mitophagy preserves cellular homeostasis by selectively degrading damaged mitochondria characterized by excessive mtROS production and reduced membrane potential. This pathway is driven by the accumulation of PTEN-induced putative kinase 1 (PINK1) on the mitochondrial outer membrane (MOM), activating the E3 ubiquitin ligase parkin, which catalyzes the assembly of ubiquitin chains on MOM proteins. This recruits autophagy adaptor proteins like optineurin (OPTN) and sequestosome 1 (SQSTM1/p62) to envelop and degrade aberrant mitochondria, ultimately reducing intracellular mtROS levels and restoring membrane potential [14]. Therefore, the induction of mitophagy to eliminate intracellular mtROS has emerged as a crucial survival strategy for intracellular pathogens. Vibrio cholerae modifies mitochondrial subcellular localization through the secretion of VopE, whereas Listeria monocytogenes triggers fragmentation of the mitochondrial network during infection, co-opting mitophagy to clear intracellular mtROS [15, 16]. Studies have shown that mitophagy occurs during *S. aureus* intracellular infection; however, whether mitophagy facilitates the survival of *S. aureus* in macrophages and the factors influencing these mitophagy alterations remain largely undetermined [17]. In our study, we discovered that *S. aureus* not only survives within macrophages but also induces mitophagy in host cells to eliminate mtROS, closely correlating with an abnormal increase in endogenous Interleukin 10 (IL10).

Previous studies have shown that the expression of the anti-inflammatory cytokine IL10 is stringently controlled by histones. Histone acetyltransferases modulate the acetylation levels at lysine residues in histone tails, which in turn influences the compactness of chromatin and consequently regulates gene promoter activity [18, 19]. Histone deacetylase 11 (HDAC11) plays a critical role in immune cells, regulating IL10 transcription by modifying acetylation levels of H3 histones and consequently inducing IL10 expression [20]. IL10 mitigates pro-inflammatory factors and resolves LPS-induced inflammatory responses via mitophagy [21, 22]. Macrophages deficient in IL10 tend to experience excessive inflammation, resulting in the accumulation of dysfunctional mitochondria and subsequent cell death [23]. However, excessive expression of IL10 can result in immune dysregulation,

thus impairing normal immune functions. Mycobacterium tuberculosis induces macrophages to produce IL10, which weakens phagosome maturation and consequently leads to chronic lung infections [24]. Biofilms of *S. aureus* can alter H3ac levels in immune cells, thereby enhancing IL10-mediated suppression of immune cells' bactericidal functions [19]. Consequently, HDAC11/IL10 likely plays a pivotal regulatory role in the intracellular survival of pathogens, making the investigation of its interaction with *S. aureus*'s intracellular persistence highly significant.

In this study, we demonstrate the importance of HDAC11 in boosting macrophage mtROS production to eliminate intracellular *S. aureus*. *S. aureus* utilizes HDAC11 to enhance IL10 transcription, thereby facilitating mitophagy to clear mtROS, minimize mitochondrial damage, and sustain an ecological niche conducive to bacterial survival (Fig. 1). These findings elucidate the mechanisms by which intracellular *S. aureus* infection triggers osteomyelitis recurrence, suggesting that targeting HDAC11 and mitophagy could significantly advance the treatment of *S. aureus* infections and osteomyelitis.

## Materials and methods

### Ethics statement

The study utilized male C57BL/6 mice, aged 6–8 weeks, acquired from Beijing Vitalstar Biotechnology Co., Ltd. All animal procedures were carried out in compliance with the International Guide for the Care and Use of Laboratory Animals and were authorized by the Research Ethics Committee of the Third Military Medical University (Chongqing Permit No. 2011-04). All surgical interventions were conducted under sodium pentobarbital anesthesia, with stringent measures taken to minimize suffering.

### Antibodies and reagents

The antibodies used in this study include: anti-Hdac11 (CST, #58442), anti-H3 (CST, #4499), anti-GAPDH (CST, #97166), anti-H3K9ac (CST, #9649), anti-mTOR (CST, #2983), anti-p-mTOR (CST, #5536), anti-β-tubulin (CST, #86298), anti-β-actin (CST, #3700), anti-Parkin (CST, #32833), anti-SQSTM1/p62 (CST, #39749), anti-LC3 (Abclone, A19665), anti-TOM20 (Abclone, A19403), anti-COX IV (Beyotime, AC610), anti-Pink1

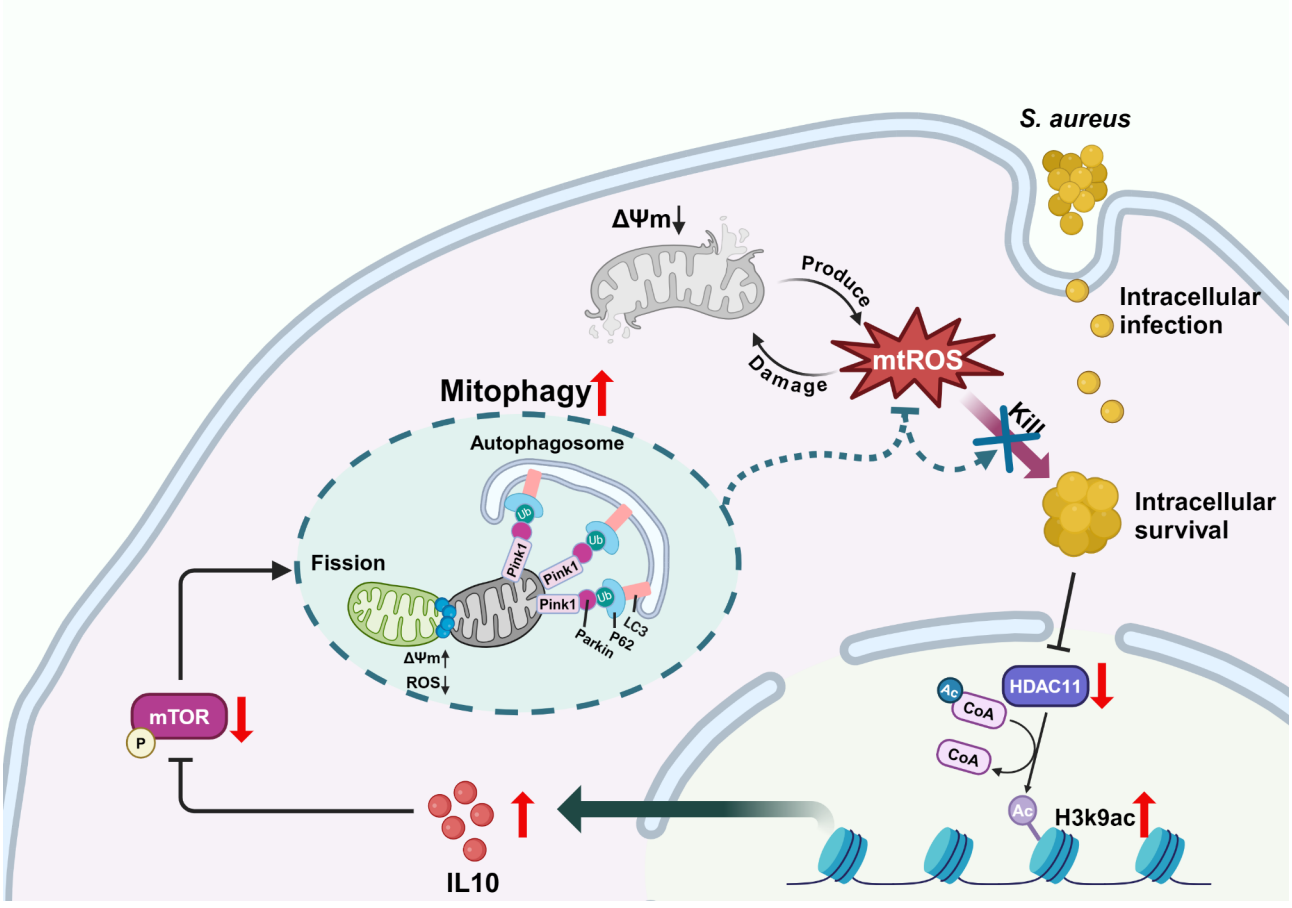


**Fig. 1** *S. aureus* suppresses HDAC11-dependent deacetylation at Histone H3 K9, enhancing IL10 transcription and translation. IL10 subsequently inhibits mTOR activation, fosters mitophagy, clears mtROS, and supports the intracellular survival of the bacteria. Created with BioRender.com

(Proteintech, 23274-1-AP). The reagents used in this study include: MitoSOX Green (Invitrogen, M36006), Lyso-tracker Green (Invitrogen, L7526), Annexin V-FITC Apoptosis Detection Kit (Beyotime, C1062M), Mitotracker Red (Beyotime, S0061M), Mitochondrial membrane potential assay kit with JC-1 (MedChem Express, HY-K0601), Mdivi-1 (MedChem Express, HY-15886), Rapamycin (MedChem Express, HY-10219), IL10 (MedChem Express, HY-P70517).

#### Bacterial strain and culture conditions

*S. aureus* (NCTC8325) was incubated overnight in 20 mL of Trypticase Soy Broth (OXOID) on a shaker at 37 °C, reaching the logarithmic growth phase, and subsequently washed three times with phosphate-buffered saline (PBS). The bacterial concentration was determined at 600 nm using a spectrophotometer and then diluted to the required CFU using PBS. When required, experiments utilized *S. aureus* (GFP-NCTC8325), engineered via homologous recombination to express green fluorescent protein (GFP) [7].

#### Cell culture and macrophage infection

Primary macrophages were obtained from the bone marrow-derived macrophages (BMDMs) of C57BL/6 mice, following the methods described in earlier literature [25]. After euthanizing the mice, bone marrow was flushed from the femurs and tibias using ice-cold PBS with a 26G needle and subsequently washed. The bone marrow was filtered through a 70 μm cell strainer (Falcon) to obtain a single-cell suspension. Immature monocytes were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% penicillin/streptomycin (Biosharp), and 10 ng/mL M-CSF (PeproTech) at 37 °C in a 5% CO2 environment. The culture medium containing 10 ng/mL M-CSF was refreshed every three days, and BMDM cells were harvested on day seven. The induced BMDM cells were stained with F4/80 (Elabscience, E-AB-F0995D) and analyzed for cell purity using flow cytometry. The results demonstrated that macrophages obtained through this method had high purity, exceeding 95% (Figure S1).

Cells were seeded in culture dishes 24 h before infection and infected with *S. aureus* or GFP-*S. aureus* at multiplicity of infection (MOI) of 10. Two hours later, 200 μg/ml gentamicin was introduced for 2 h to remove extracellular free bacteria, followed by three PBS washes. The culture medium (RPMI-1640 with 10% fetal bovine serum and 1% penicillin/streptomycin) containing 50 μg/mL gentamicin was added to inhibit the growth of extracellular bacteria, marking this time point as 0 h post-intracellular infection.

#### Intracellular bacterial survival

To determine the intracellular bacterial load, 2 × 10^6 BMDMs were seeded in 100 mm culture dishes and grown overnight, followed by *S. aureus* infection of macrophages as previously described. Following the designated infection period, supernatants were discarded, cells were washed twice with PBS, and lysed using PBS containing 0.1% Triton-X. Serial dilutions were plated on tryptic soy agar and incubated at 37 °C for 24 h, followed by colony counting. To explore the impact of the mitophagy inhibitor Mdivi-1 and the mammalian target of rapamycin (mTOR) inhibitor rapamycin on intracellular bacterial survival, cells were pre-treated with Mdivi-1 (20 μM) or rapamycin (100 nM) for 2 h prior to experimentation. For plasmid and siRNA transfection, BMDMs were seeded at a density of 2 × 10^5 cells per well in six-well plates overnight. Cells were transfected with 2.5 μg pDONR223-HDAC11 plasmid (purchased from YouBio, Changsha, China) using Lipofectamine 3000 (Invitrogen) and subjected to experiments 24 h post-transfection. The IL10 silencing RNA (siRNA) was purchased from TSINGKE (Wuhan, China) and transfected into cells using Lipofectamine 3000 according to the manufacturer's instructions (final siRNA working concentration: 30 nM/mL). For subsequent experiments, IL10 (1 ng/mL) was additionally added to BMDMs treated with siRNA and Mdivi-1.

#### Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from 1 × 10^7 BMDM cells in each treatment group using an RNA extraction kit (Invitrogen). cDNA was obtained using the ABScript II One Step SYBR Green RT-qPCR Kit (Abclone), followed by analysis using qRT-PCR (CFX Connect, BIO-RAD, United States). The relative levels of mRNA expression were calculated using the comparative CT method (ΔΔCt). The primer sequences used were: IL10(Forward: CAGAGAAGCATGGCCCAGAAATC; Reverse: GCTC CACTGCCTTGCTCTTATTT), GAPDH(Forward: ACT CTTCCACCTTCGATGCC; Reverse: TGGGATAGGGC CTCTCTTGC).

#### Western Blot与Enzyme-linked immunosorbent assay(ELISA)

As previously described, BMDMs were pre-cultured in culture dishes and co-transfected with plasmids or siRNA as needed, or exposed to stimulation with Mdivi-1 or rapamycin. Following infection with *S. aureus*, cells were harvested and lysed using RIPA Lysis Buffer containing protease and phosphatase inhibitors, and total proteins were isolated by centrifuging the lysates at low temperature to collect the supernatant. Proteins were separated using SDS-Polyacrylamide Gel Electrophoresis

and subsequently transferred onto polyvinylidene difluoride (PVDF) membranes. PVDF membranes were blocked with 5% skim milk for one hour and then incubated overnight at 4 °C with specific primary antibodies. Subsequently, the membranes were thoroughly washed with TBST (Tris Buffered Saline with Tween-20) and incubated for one hour at room temperature with either anti-mouse or anti-rabbit IgG. We used an enhanced chemiluminescence detection kit (Introvigen) to detect protein bands on the membrane, utilizing the chemiluminescence imaging system (ChemiDoc MP Imaging System, BIO-RAD, United States). The grayscale values were analyzed using ImageJ software. Mitochondrial proteins were isolated for analysis using a Mitochondria Isolation Kit (Beyotime).

For ELISA, the levels of inflammatory factors in the culture supernatant of stimulated or unstimulated BMDMs were measured according to the manufacturer's instructions using IL6, TNF-α, IL-1β, IL12, and TGF-β ELISA kits (Dakewe Biotech Co., 1210602, 1217202, 1210122, 1211202, 1217102) and the IL10 ELISA kit (Beyotime, PI522).

#### Co-immunoprecipitation (Co-IP)

Co-IP assays were performed using the Beyotime protocol. Antibodies against HDAC11, H3K9ac, and IgG were thoroughly mixed with magnetic beads and incubated overnight at 4 °C with rotation. Treated cells were lysed using RIPA lysis buffer. The lysates were centrifuged (4 °C, 12,000×g, 10 min), and the supernatants were collected and divided into three groups: Input, IgG, and IP. Protein concentrations were measured and stored for further use. The magnetic bead-antibody complexes (HDAC11 or H3K9ac antibodies) were added to the IP group supernatant, while the magnetic bead-IgG complex was added to the IgG group supernatant. The mixtures were incubated at room temperature for 4 h. The bead-antibody-protein complexes were separated using a magnetic stand and washed three times with PBS. SDS-PAGE loading buffer (1×) was added to each group (Input, IgG, and IP), followed by heating at 95 °C for 5 min. The supernatants were collected after magnetic separation and subjected to WB analysis.

#### Bioinformatics analysis

As previously described, 2 × 10^6 BMDMs were seeded in 100 mm culture dishes and grown overnight. The total protein from *S. aureus* (MOI = 10) infected BMDMs (24-hour infection) or uninfected BMDMs was subjected to quantitative proteomic analysis. Samples were submitted to Panomix Biotechnology Co., Ltd. (China) for protein identification. LC-MS/MS analysis was performed using the Thermo Scientific Orbitrap Exploris™480 system, equipped with a nano electrospray ion source and coupled to an EASY nLC 1200 ultra-high-pressure system (Thermo Fisher Scientific). Proteins with p-values ≤ 0.05 and |log2(fold change)| ≥1 were identified as differentially expressed and analyzed for heatmap generation, volcano plots, GO enrichment, and KEGG pathway enrichment using OmicShare tools, a free online platform (https://www.omicshare.com/tools).

#### Flow cytometry

2 × 10^6 BMDMs were pre-seeded in 100 mm culture dishes and incubated overnight. The cells were then infected with GFP-*S. aureus* (MOI = 10) for 2 h, followed by co-incubation with gentamicin for a designated period. The culture medium was subsequently removed, cells were washed with PBS, and then collected by centrifugation. Intracellular bacteria display green fluorescence at a 488 nm excitation wavelength, which facilitates the monitoring of bacterial growth through the analysis of intracellular fluorescence intensity and positive cell populations.

MitoSOX Green is employed to evaluate mtROS levels. Following the manufacturer's instructions, dilute the MitoSOX Green stock solution with PBS to a 1 μM working concentration, incubate the cells with this solution at 37 °C in the dark for 30 min, then collect the cells for analysis via flow cytometry at 488 nm.

To measure mitochondrial membrane potential using the JC-1 assay, dilute the JC-1 stock solution to a working concentration of 2 μM in complete culture medium. Mix thoroughly with the cells, incubate at 37 °C for 20 min, then resuspend in PBS. Fluorescence intensities at green (510/527 nm) and red (585/590 nm) wavelengths are monitored using flow cytometry.

Cell apoptosis was assessed using the Annexin V-FITC Apoptosis Detection Kit. Briefly, post-treatment cells were collected and washed twice with PBS. Subsequently, 195 μL of binding buffer was added to the treated cells. Each sample then received 5 μL of annexin V-FITC and 10 μL of PI and was incubated in the dark at room temperature for 15 min. Flow cytometry analysis was conducted using a BD FACSVerse system (BD Biosciences, USA). Data collection and analysis were performed using FlowJo software.

#### Confocal laser scanning microscopy (CLSM)

1 × 10^5 BMDMs were seeded in 20 mm confocal culture dishes and incubated overnight. The cells were then infected with *S. aureus* at MOI = 10 for 2 h. Cells were washed 2–3 times with PBS, then incubated in complete medium containing gentamicin to eliminate extracellular bacteria for a designated period. After washing the cells three times with PBS, the cell membranes were stained using Dil dye (Beyotime). Cells were fixed for 20 min using immunofluorescence fixative and subsequently

imaged with CLSM (Leica TCS SP8, Germany), where live *S. aureus* exhibited green fluorescence at an excitation wavelength of 488 nm.

For assessing mitochondrial membrane potential, cells were treated with JC-1 dye at 37 °C for 20 min as per manufacturer's instructions, and then observed using CLSM. Colocalization of mitochondria and lysosomes serves as an indicator of mitophagy. BMDMs were incubated with 20 nM Mito-Tracker for 30 min, followed by 2–3 washes with PBS. Subsequently, cells were incubated with 50 nM Lyso-Tracker for 5 min, followed by 2–3 PBS washes. After staining nuclei with Hochest dye (Beyotime) for 30 min, cells were placed in fresh culture medium for observation. Analysis was conducted using ImageJ software (NIH, Bethesda, MD).

#### Chromatin immunoprecipitation (ChIP) assay followed by qPCR (ChIP-qPCR)

Chromatin immunoprecipitation assays were performed according to the previously described [26]. In brief, treated cells were washed twice with cold PBS buffer and cross-linked with 1% formaldehyde at room temperature for 10 min, followed by the addition of glycine (final concentration 125 mmol/L) to terminate the reaction. The cells were then lysed, and chromatin was isolated on ice. The chromatin was sonicated to obtain soluble sheared chromatin (average DNA length of 200–500 bp). A 20 μL aliquot of chromatin was stored at − 20 °C as input DNA, and 100 μL of chromatin was used for immunoprecipitation with anti-H3K9ac antibody (CST, #9649) or IgG antibody (CST, #2729s). For immunoprecipitation, 10 μg of antibody was incubated with the sample overnight at 4 °C. The samples were then incubated with protein G beads (Thermofisher, #10004D) for 3 h. The beads were washed once with 20 mM Tris-HCl (pH 8.1), 50 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 0.1% SDS; twice with 10 mM Tris-HCl (pH 8.1), 250 mM LiCl, 1 mM EDTA, 1% NP-40, and 1% sodium deoxycholate; and twice with 1× TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA). The bound chromatin was eluted in 300 μL of elution buffer (100 mM NaHCO3, 1% SDS). The eluted samples were treated with RNase A (final concentration 8 μg/mL) at 65 °C for 6 h, followed by proteinase K (final concentration 345 μg/mL) digestion at 45 °C overnight. The enrichment of IL10 promoter regions was evaluated using ChIP primers for IL10 by qRT-PCR as previously described, with enrichment values normalized to the input samples and calculated using the 2^(-ΔΔCt) method. IL10 promoter primers: Forward: ATCTAGAG AGTCCTAGGGAAAGC; Reverse: AGATCTAGAGACT ACGCAGAGAC.

#### Statistical analysis

All experiments were conducted in three independent repeats to ensure data reliability. Data are presented as the mean ± standard deviation (SD) from experimental replicates. One-way analysis of variance (ANOVA) or Student's t-tests were employed to assess the statistical significance of intergroup differences. Data analyses and graphical representations were generated using Prism software (version 8.0; GraphPad). A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

## Results

#### *S. aureus* infects macrophages and maintains intracellular persistence via IL10

To explore the intracellular persistence of *S. aureus* in macrophages, we infected BMDMs with GFP-*S. aureus* during its logarithmic growth phase (MOI = 10). Two hours post-infection, extracellular bacteria were eliminated and the growth of extracellular *S. aureus* was suppressed using complete medium supplemented with antibiotics. Following bacterial clearance, intracellular culture times were recorded, and images capturing bacterial proliferation were taken at various time points. Confocal microscopy imaging revealed that despite the presence of extracellular antibiotics, intracellular *S. aureus* actively proliferated within BMDMs over 24 h (Fig. 2A, B). Additionally, we employed the same methodology to establish an intracellular survival model for *S. aureus*, leveraging the fluorescence properties of GFP-*S. aureus* to assess bacterial proliferation within individual cells and the proportion of infected cells via flow cytometry [27]. Results indicated that with prolonged infection, the fluorescence intensity within individual cells increased, as did the proportion of positive cells (Fig. 2C, D). This provides additional evidence of *S. aureus*'s ability to proliferate within macrophages over extended periods.

Therefore, we evaluate the impact of intracellular *S. aureus* infection on BMDMs by analyzing cell apoptosis and the expression levels of pro-inflammatory and anti-inflammatory factors, to determine if these cells maintain their normal immune functionality. Employing the same procedure, BMDMs were infected with *S. aureus* and, following the removal of extracellular bacteria, cells were collected at various time points for flow cytometry analysis to assess apoptosis. We observed that despite the continuous proliferation of *S. aureus* within BMDMs over 24 h, there was no substantial increase in the rate of apoptosis or necrosis in the host cells compared to the initial infection time (0 h) and control groups (Fig. 2E, F, G). Supernatants were collected for cytokine ELISA analysis, which showed that BMDMs infected with *S. aureus* for 24 h exhibited increased expression of the pro-inflammatory cytokines TNF-α, IL1β, and IL6, consistent with prior research [28]. Interestingly, BMDMs infected

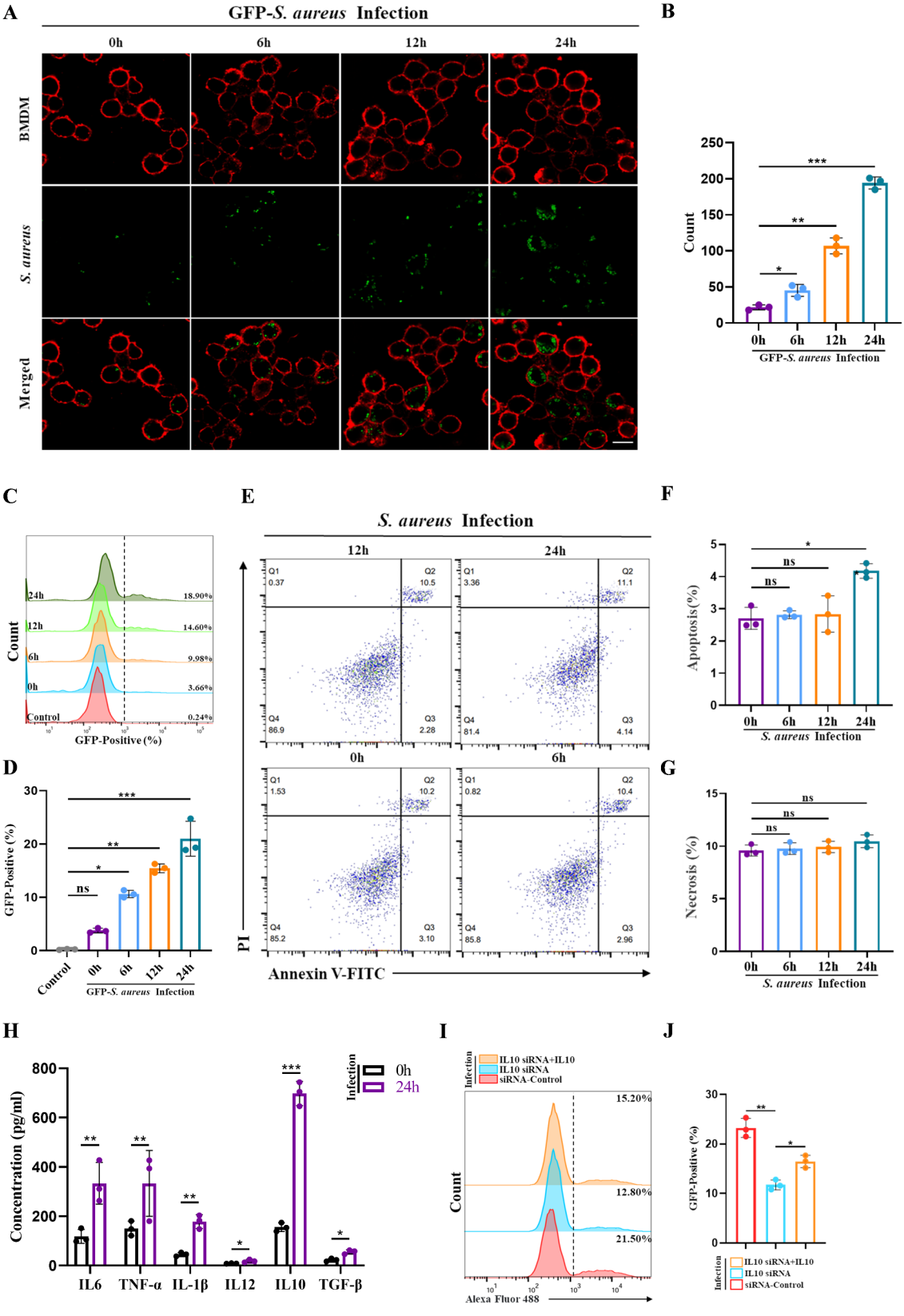


**Fig. 2** (See legend on next page.)

(See figure on previous page.)

**Fig. 2** *S. aureus* infects macrophages and maintains intracellular persistence via IL10. **(A** and **B)** Confocal microscopy images showing the growth of *S. aureus* (green) within BMDMs (red) at various time points in the presence of antibiotics (MOI = 10) (Scale bar: 10 μm). Quantitative analysis of intracellular bacterial counts at each time point. **(C** and **D)** Flow cytometry analysis of the fluorescence intensity within BMDMs infected with GFP-*S. aureus* (MOI = 10) over different time points. Quantitative analysis of the proportion of BMDMs infected with GFP-S. aureus among groups. **(E, F** and **G)** Flow cytometry analysis of apoptosis in BMDMs infected with *S. aureus* (MOI = 10) at various time points after treatment with Annexin V-FITC/PI. Quantitative analysis of apoptosis and necrosis rates in BMDMs following intracellular infection with *S. aureus* **(H)** ELISA quantification of IL6, TNF-α, IL1β, IL12, IL10, and TGF-β levels in the supernatant of BMDMs infected with *S. aureus* (MOI = 10) at 0 and 24 h. **(I** and **J)** Flow cytometry analysis of intracellular bacterial growth in BMDMs 24 h after infection with GFP-*S. aureus* (MOI = 10), following transfection with control siRNA or IL10 siRNA and in the presence or absence of added IL10 (1ng/ml). Quantitative analysis of the proportion of BMDMs infected with GFP-*S. aureus* among groups. Statistical analysis was performed using one-way ANOVA **(B, D, F, G)** or two-tailed Student's unpaired t-test **(H, J)**: ns, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The data represent the mean ± SD from at least three independent experiments

with *S. aureus* for 24 h significantly upregulated the anti-inflammatory cytokine IL10, whereas TGF-β, another key anti-inflammatory cytokine in macrophages, did not show significant changes. Additionally, the increase in IL10 was significantly more pronounced than that observed in other anti-inflammatory cytokines (Fig. 2H). To explore IL10's impact on the intracellular survival of *S. aureus* in macrophages, we employed siRNA to suppress IL10 production. Post-suppression, there was a significant reduction in endogenous IL10 in BMDMs after they were intracellularly infected with *S. aureus* (Fig. S1). Furthermore, in the IL10 siRNA-treated group, intracellular growth of *S. aureus* within BMDMs was significantly inhibited within 24 h, but this inhibition was reversed upon reintroduction of IL10 (Fig. 2I, J).

These findings demonstrate that *S. aureus* can survive and proliferate within macrophages, while also inhibiting the host's inflammatory mediators and programmed apoptosis, likely facilitated by elevated IL10 expression.

### *S. aureus* triggers mitophagy in BMDMs

To delve into how *S. aureus* within macrophages impacts immune functions and contributes to intracellular proliferation, we conducted a series of proteomic studies. Employing the same modeling approach, *S. aureus* was established within BMDM for 24 h, after which quantitative proteomics were used to compare these cells with uninfected controls. By identifying proteins with $a|\log2(\text{fold change})| \geq 1$ and a p-value < 0.05, we detected 426 proteins that were upregulated and 262 that were downregulated (Fig. 3. A, B). GO enrichment analysis of differentially expressed proteins revealed significant enrichment in mitochondrial components within the Cellular component category, particularly in the mitochondrial matrix and inner mitochondrial membrane, among other mitochondria-related pathways. Additionally, the differentially expressed proteins were enriched in biological processes including mitochondrial gene expression, selective autophagy, and the positive regulation of IL10 production, further substantiating our earlier findings. KEGG pathway enrichment analysis indicated significant enrichment of these proteins in metabolic and autophagy pathways (Fig. 3. C, D). Therefore, we hypothesize that intracellular *S. aureus* infection mediates alterations in mitochondrial function and autophagy levels, facilitating the bacterium's survival within macrophages.

To validate this hypothesis, we further investigated the mitochondrial function in BMDMs intracellularly infected with *S. aureus*. mtROS, a crucial component of macrophages' antimicrobial arsenal, primarily arises from the mitochondrial oxidative phosphorylation process [9, 29]. Using MitoSOX staining, we observed that mtROS levels were significantly elevated in the early stages of intracellular *S. aureus* infection; however, this induction was not sustained. As *S. aureus* persisted within the cells, the levels of mtROS progressively decreased (Fig. 3. E, F). mtROS is a primary driver of oxidative stress and mitochondrial membrane potential loss in cells [30]. Using JC-1 staining and confocal microscopy, we observed that an increase in mtROS levels correlates with damage to mitochondrial membrane potential in host cells. However, with the progression of the infection, mitochondrial membrane potential levels gradually recovered (Fig. 3. G, H). This observation was further validated by flow cytometry analysis (Fig. 3. I, J). Additionally, we employed confocal microscopy to analyze the colocalization of mitochondria and lysosomes. The results demonstrated that *S. aureus* infection significantly increased the colocalization of mitochondria and lysosomes, suggesting potential mitochondrial degradation by lysosomes.

The findings indicate that *S. aureus* can modify mitochondrial function in BMDMs and inhibit ongoing mtROS-induced damage to both itself and the host cell mitochondria, thereby preserving its intracellular niche. This alteration is likely due to changes in the mitophagy process.

### IL10 enhances intracellular bacterial survival by modulating mitophagy to suppress mtROS

Mitophagy necessitates the initiation of mitochondrial fission, with dynamin-related protein 1 (DRP1) serving as the principal regulatory factor [31]. Mdivi-1 significantly suppresses mitophagy by negatively regulating DRP-1. After pre-treating BMDM cells with Mdivi-1, we observed that the intracellular growth of *S. aureus* was significantly reduced compared to the untreated control.

The bacterial load continued to decline 12 h after infection (Fig. 4. A, B). This effect was similarly confirmed through flow cytometry analysis (Fig. 4. C, D). Flow cytometry data revealed that treatment with Mdivi-1 led to a sustained increase in intracellular mtROS levels, enabling cells to significantly resist ongoing intracellular proliferation of *S. aureus*(Fig. 4. E, F). Mitochondrial proteins and total cell proteins were extracted from BMDMs at 6, 24 h post-infection for Western blot analysis. Results indicated that intracellular *S. aureus* infection significantly induced the recruitment of microtubule-associated protein 1 A/1B-light chain 3 (MAP1LC3) and p62 to mitochondria, facilitating lysosome-mediated selective autophagy of mitochondria. The level of mitochondrial marker translocase of outer mitochondrial membrane 20 (TOM20) was also found to decrease in whole cell lysates during the infection process. Mdivi-1 treatment significantly inhibited the recruitment of autophagy proteins to mitochondria and the reduction in total TOM20 levels induced by *S. aureus* infection (Fig. 4. G, H, I, J). These findings demonstrate that inhibiting mitophagy effectively restores and sustains increased mtROS levels in BMDM within 24 h of *S. aureus* infection, which enhances the clearance of intracellular bacteria.

Studies indicate that IL10 induces the production of an mTOR inhibitor called DDIT4 to suppress mTOR activity, promoting mitophagy to clear accumulated intracellular mtROS. By silencing IL10 expression using siRNA, we observed that 24 h after *S. aureus* infection, intracellular mtROS levels in the treated group were significantly higher than in the untreated group, but were reduced upon supplementary IL10 administration. To further confirm the role of IL10 in enhancing mitophagy and its impact on intracellular *S. aureus* survival, we supplemented cells with mitophagy inhibited by Mdivi-1 with IL10. We observed an enhancement in *S. aureus* growth compared to the group treated solely with Mdivi-1 (Fig. 4. K, L). Flow cytometry analysis of mtROS showed that the addition of IL10 mitigated the increase in mtROS induced by Mdivi-1-mediated inhibition of mitophagy (Fig. 4. M, N). This led to a significant reduction in intracellular mtROS levels, thereby enhancing the survival of intracellular *S. aureus* (Fig. 4. O, P). These findings demonstrate that during *S. aureus* infection in BMDMs, the elimination of mtROS through mitophagy mediated by host cell IL10 is critical for the bacteria's intracellular survival.

### *S. aureus* infection results in the downregulation of HDAC11 in macrophages, affecting the regulation of IL10 and mitophagy

Analysis of differentially expressed proteins revealed a significant decrease in HDAC11 expression in BMDMs 24 h post-*S. aureus* infection **(**Fig. 5. **A)**. Studies have found that HDAC11 is a transcriptional regulator of IL10, and overexpression of HDAC11 inhibits the transcriptional activity of IL10 [32, 33]. Using Western blotting, we observed that HDAC11 levels in macrophages did not significantly decrease during the initial 0 to 6 h of intracellular *S. aureus* infection; however, HDAC11 levels declined over the course of the infection from 6 to 24 h (Fig. 5. B, C). Studies have reported that the acetylation of lysine 9 on histone H3 (H3K9ac) at the IL10 promoter plays a regulatory role in the transcriptional expression of IL10 [34]. Therefore, we examined the changes in H3K9ac during *S. aureus* intracellular infection and its enrichment at the IL10 promoter. We found that H3K9ac levels increased as HDAC11 decreased, accompanied by an increased enrichment of H3K9ac at the IL10 promoter and an upregulation of IL10 transcription (Fig. 5. B, D, S3).This is consistent with the trend of IL10 changes shown in the ELISA results (Fig. 5E). The Co-IP results showed that HDAC11 can directly bind to H3K9ac, exerting its deacetylation regulatory function on H3K9ac (Fig. S4).

As previously described, IL10 modulates mitophagy via mTOR, and proteomic analysis has shown significant changes in the autophagy protein SQSTM1/P62 in BMDMs following infection. Consequently, we investigated changes in autophagy levels during the 24-hour intracellular survival of *S. aureus* in BMDMs. LC3 and P62 are critical proteins involved in autophagy. The conversion of LC3 from LC3-I to LC3-II marks autophagosome formation, and P62 is commonly degraded during the autophagic process. Compared to the control group, the LC3II/LC3I ratio significantly increased at both 12 and 24 h, while P62 levels significantly decreased at these intervals, indicating pronounced activation of autophagy starting 6 h post-*S. aureus* infection. mTOR is a key regulatory factor for DRP1, which initiates mitophagy [35] 。 We observed a significant decrease in the p-mTOR/mTOR ratio beginning 6 h post-infection (Fig. 5. F, G, H, I). PINK1-Parkin are key proteins that regulate mitophagy. Levels of PINK1 and Parkin increased relative to controls from 0 to 6 h post-infection, with significant elevations observed between 12 and 24 h (Fig. 5. J, K, L). This corroborates previous findings that intracellular *S. aureus* infection stimulates mitophagy.

These studies suggest that during the persistent survival of *S. aureus* within macrophages, the increase in mitophagy mediated by IL10 may result from the inhibition of HDAC11 in host cells, leading to elevated H3K9ac levels and increased enrichment at the IL10 promoter region, thereby promoting IL10 transcription.

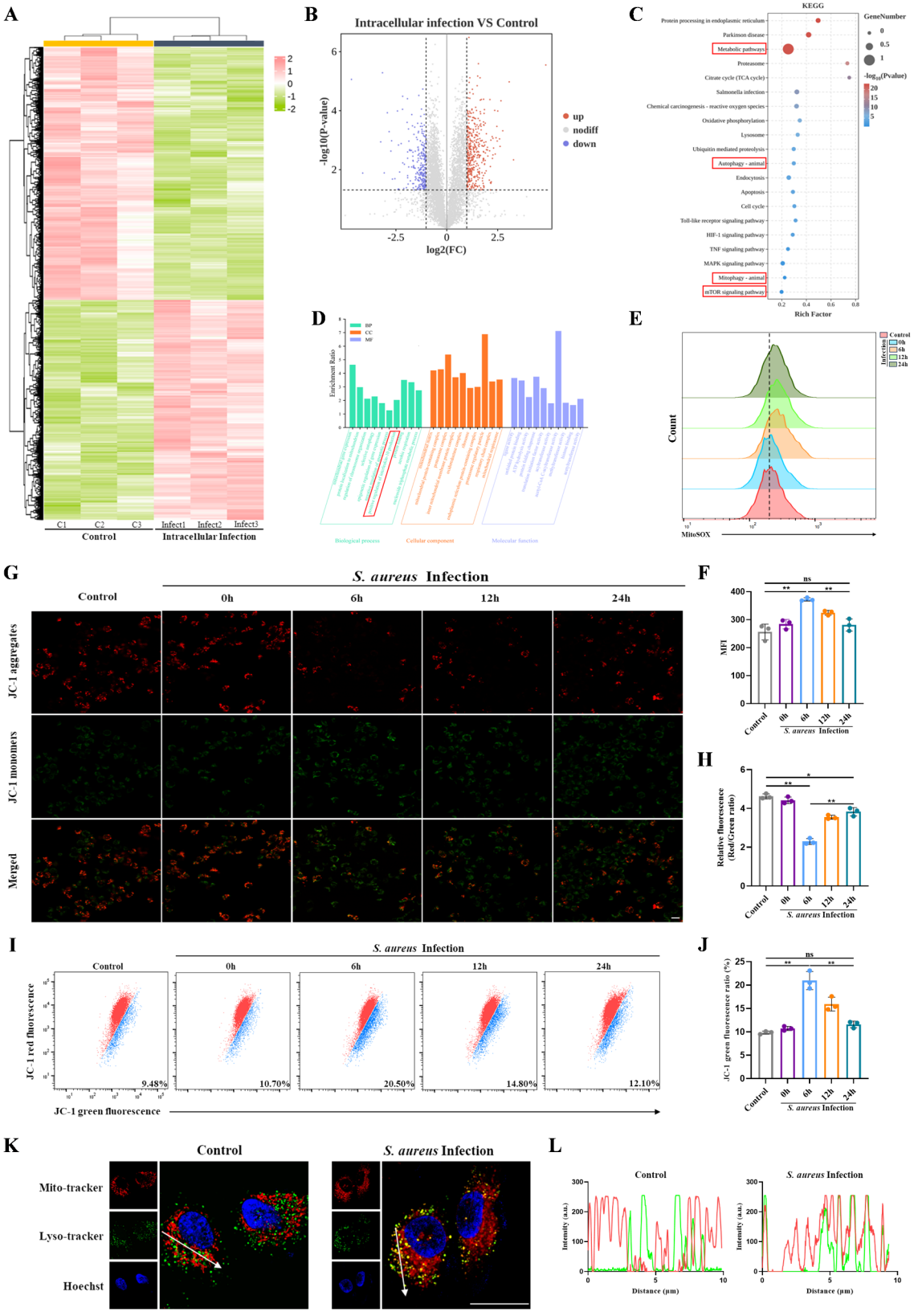


**Fig. 3** (See legend on next page.)

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**Fig. 3** *S. aureus* triggers mitophagy in BMDMs. **(A)** Heatmap displaying clustered global protein expression profiles ($n = 3$). **(B)** Volcano plot comparing differential protein expression between BMDMs infected with *S. aureus* for 24 h and uninfected groups. Proteins with| log2(fold change)| ≥1 and $P < 0.05$ are identified as significantly differentially expressed. **(C)** Bubble plot derived from KEGG pathway enrichment analysis. The enrichment factor indicates the degree of enrichment, while larger p-value (-log10) denote higher statistical significance. Larger bubbles signify greater enrichment levels. **(D)** GO enrichment analysis for proteins expressed differentially. The enrichment factor quantifies the level of enrichment. **(E** and **F)** Flow cytometry analysis of mtROS levels in BMDMs infected with *S. aureus* (MOI = 10) at various time points. Quantitative assessment is based on mean fluorescence intensity (MFI) indicative of intracellular mtROS levels. **(G** and **H)** Using confocal microscopy to observe changes in mitochondrial membrane potential (ΔΨm) in BMDMs infected with *S. aureus* (MOI = 10), at various time points after JC-1 staining. Red fluorescence indicates JC-1 aggregates, reflective of intact mitochondrial membrane potential, while green fluorescence signifies JC-1 monomers, indicating ΔΨm dissipation (Scale bar: 10 μm). Quantitative analysis of the red/green fluorescence intensity ratio reflects the overall state of mitochondrial membrane potential within the cells. **(I** and **J)** Employing JC-1 staining followed by flow cytometry to assess changes in ΔΨm. The analysis quantifies the percentage of cells positive for JC-1 monomers. **(K** and **L)** Confocal microscopy images depicting mitochondria (Mito-tracker, red), lysosomes (Lyso-tracker, green), and nuclei (Hochst, blue) in BMDMs (Scale bar: 10 μm). Histogram profiles are created by quantifying the fluorescence intensity along designated paths in the red (Mito-tracker) and green (Lyso-tracker) channels. Statistical analysis was performed using one-way ANOVA: ns, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The data represent the mean ± SD from at least three independent experiments

### HDAC11 regulates mitophagy via the IL10/mTOR pathway to clear mtROS, crucial for intracellular bacterial survival

We transfected BMDMs with an HDAC11 overexpression (OE-HDAC11) plasmid and observed a significant upregulation of HDAC11 protein levels. Meanwhile, the H3K9ac levels decreased, supporting the role of HDAC11 in regulating the acetylation of H3K9 (Fig. 6. A, B, C). ChIP-qPCR analysis revealed that in HDAC11-overexpressing BMDMs following *S. aureus* intracellular infection, the enrichment of H3K9ac at the IL10 gene promoter was reduced, accompanied by suppressed IL10 transcription levels (Fig. S5). ELISA analysis revealed that overexpressing HDAC11 significantly decreased IL10 levels in BMDMs and effectively mitigated the rise in IL10 triggered by intracellular *S. aureus* infection **(**Fig. 6. **D)**. Therefore, we confirmed that HDAC11 regulates IL10 transcription by controlling H3K9ac levels and its enrichment at the IL10 promoter.

IL10 is known to activate mTOR, thereby enhancing autophagy and mitophagy. Rapamycin, an autophagy inducer, boosts cellular autophagy and mitophagy by inhibiting mTOR phosphorylation [36]. Utilizing rapamycin to modulate mTOR, combined with HDAC11 overexpression, we assessed whether HDAC11, through IL10, influences intracellular bacterial survival by targeting mTOR. Employing bacterial plating (Fig. 6. E, F) and flow cytometry (Fig. 6. G, H) to assess intracellular bacteria, we found that *S. aureus* proliferation was suppressed in BMDMs following plasmid transfection; however, rapamycin pre-treatment led to significant bacterial proliferation within 24 h, counteracting the growth inhibition induced by HDAC11 overexpression. These findings suggest that the HDAC11/IL10 pathway, modulating mTOR, is critical for the intracellular survival of *S. aureus*.

Observations on mitophagy, through MitoSOX measurements of mtROS, revealed that the OE-HDAC11 group experienced a continuous rise in mtROS levels throughout the 24 h of intracellular *S. aureus* infection. And rapamycin significantly curtailed the rise in mtROS between 6 and 24 h and mitigated the heightened mtROS levels induced by HDAC11 overexpression (Fig. 7. A, B). Elevated mtROS resulted in a sustained decrease in mitochondrial membrane potential within host cells throughout the 24-hour period of intracellular *S. aureus* infection. Rapamycin mitigated the decline in mitochondrial membrane potential within 6 h of *S. aureus* infection and facilitated its recovery from 6 to 24 h. Additionally, rapamycin ameliorated the decrease in mitochondrial membrane potential induced by HDAC11 overexpression (Fig. 7. C, D). Confocal imaging confirmed that HDAC11 overexpression suppressed mitophagy induced by *S. aureus*, and rapamycin enhanced mitophagy in infected host cells and reversed the inhibition of mitophagy caused by HDAC11 overexpression (Fig. 7. E). Western Blot analysis revealed that HDAC11 overexpression reduced the expression of autophagy markers LC3 and P62, while simultaneously activating mTOR (Fig. 7. F, G, H, I); The expression of PINK1 and Parkin in mitochondria was notably decreased, inhibiting the mitophagy triggered by intracellular *S. aureus* infection (Fig. 7. J, K, L). Rapamycin enhanced mitophagy and counteracted the suppression caused by HDAC11 overexpression, facilitating *S. aureus* colonization within host cells.

In conclusion, the HDAC11/IL10 pathway, via mTOR, enhances mitophagy to clear mtROS, supporting the sustained 24-hour intracellular survival of *S. aureus*.

## Discussion

*S. aureus* is the principal pathogen causing osteomyelitis, with its resistance mutations and immune evasion mechanisms contributing to the high recurrence and challenging management of the disease [37]. Earlier research on recurrent osteomyelitis cases revealed that, under antibiotic pressure, macrophages act as a primary reservoir for *S. aureus*, offering a replication niche [7]. This study shows that the HDAC11/IL10 pathway is crucial in regulating reactive oxygen-mediated bactericidal activity during intracellular *S. aureus* infection in macrophages. Downregulation of HDAC11 enhances histone

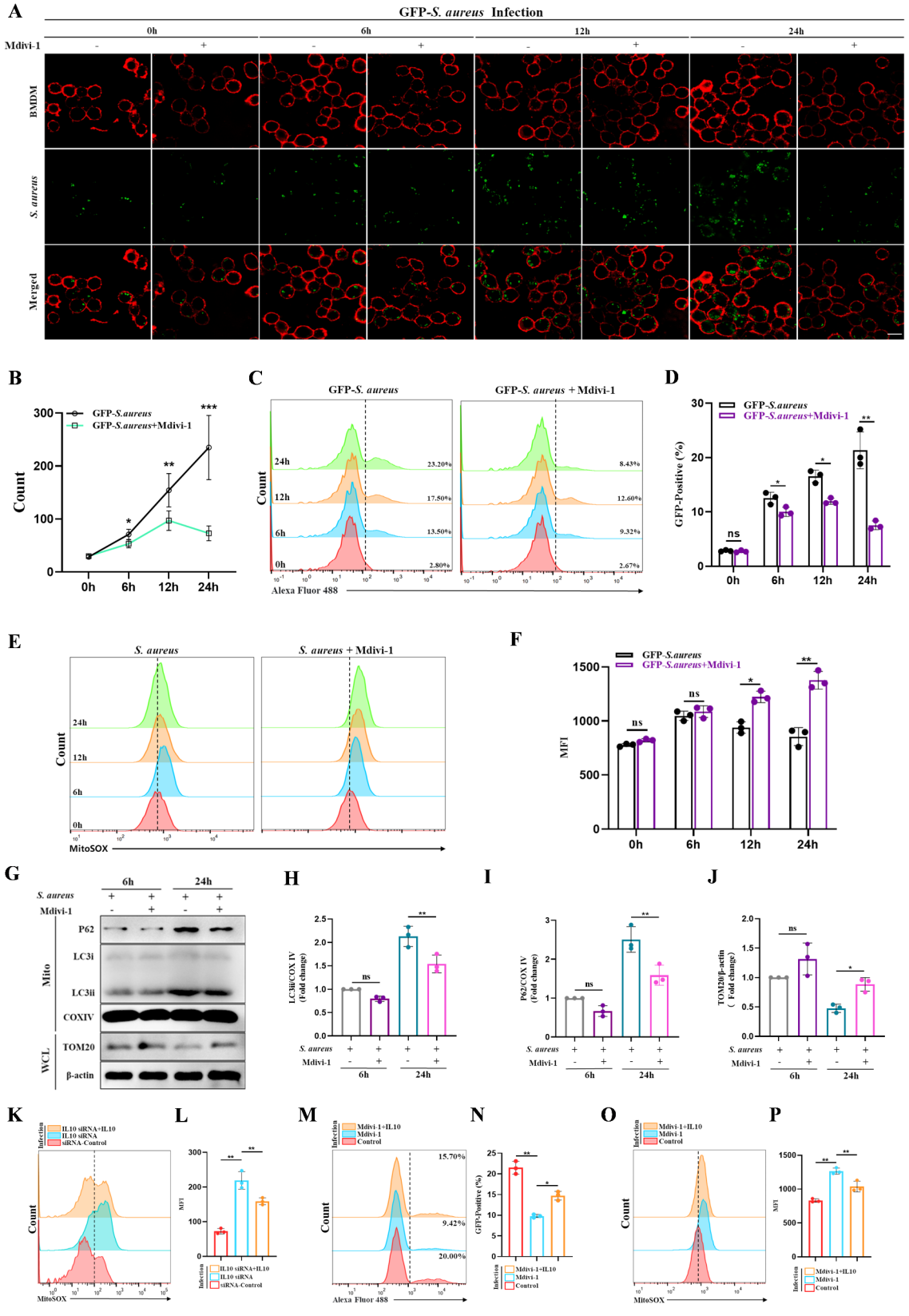


**Fig. 4** (See legend on next page.)

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**Fig. 4** IL10 enhances intracellular bacterial survival by modulating mitophagy to suppress mtROS. **(A** and **B)** Confocal microscopy demonstrating the effect of the mitophagy inhibitor Mdivi-1 (20μM) on the bacterial load within BMDM cells (Scale bar: 10 μm). Quantitative analysis of intracellular bacterial counts at various time points. **(C** and **D)** Flow cytometry analysis of the effect of Mdivi-1 (20μM) on the bacterial load of *S. aureus* within BMDM cells. Quantitative analysis of the proportion of BMDM cells infected with GFP-*S. aureus* at various time points. **(E** and **F)** Flow cytometry analysis of the effects of Mdivi-1 (20μM) on mtROS production in BMDMs infected with *S. aureus* at different time points. Quantitative analysis of Mean Fluorescence Intensity (MFI) reflecting mtROS levels within BMDMs. **(G**, **H**, **I**, and **J)** After pre-treatment of BMDM cells with Mdivi-1 (20μM), followed by infection with *S. aureus* for 6 and 24 h, mitochondrial (Mito) and whole cell lysate (WCL) were prepared for Western blot analysis. COX IV served as a mitochondrial marker, and β-actin as a whole cell lysate marker. Using COX IV as a reference protein, quantitative analysis of the relative expression levels of LC3 and P62 was conducted. Relative expression levels of TOM20 were analyzed using β-actin as a reference protein. **(K** and **L)** Flow cytometry analysis of mtROS levels in BMDMs 24 h post-infection with GFP-*S. aureus* (MOI = 10), following transfection with either siRNA-Control or IL10 siRNA, with or without the addition of IL10 (1ng/ml). Quantitative analysis of Mean Fluorescence Intensity (MFI) reflects intracellular mtROS levels. **(M** and **N)** Flow cytometry analysis of the impact of Mdivi-1 (20μM) pretreatment, with or without added IL10 (1ng/ml), on the bacterial load of BMDMs infected with GFP-*S. aureus* (MOI = 10) for 24 h. Quantitative analysis of the proportion of BMDMs infected with GFP-*S. aureus*. **(O** and **P)** Flow cytometry analysis of mtROS levels in BMDMs 24 h after infection with *S. aureus* (MOI = 10) in various treatment groups. Quantitative analysis of intracellular mtROS levels. Statistical analysis was performed using a two-tailed Student's unpaired t-test: ns, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The data represent the mean ± SD from at least three independent experiments

H3 acetylation, which upregulates the anti-inflammatory factor IL10, boosts mitophagy, effectively clears mtROS, and supports the survival of *S. aureus* in macrophages beyond 24 h.

An increasing body of literature identifies *S. aureus* as a facultative intracellular pathogen capable of infecting and surviving within macrophages and neutrophils [27, 38, 39]. During osteomyelitis, *S. aureus* exploits intracellular residency in macrophages to evade antimicrobial and antibiotic attack, facilitating its spread within the host and contributing to disease recurrence [6]. This study confirms that *S. aureus* can sustain intracellular survival within macrophages for at least 24 h. As key players in innate immunity, macrophages activate inflammatory pathways via TLRs, producing numerous pro-inflammatory cytokines; they further combat infection by generating substantial amounts of ROS through mitochondria and other organelles [40, 41]. To ensure intracellular survival, *S. aureus* must counter the antimicrobial defenses of macrophages. Increasing evidence shows that intracellular *S. aureus* can inhibit host cell inflammatory responses. For instance, *S. aureus* secretes extracellular fibrinogen-binding protein, which facilitates the recruitment of TRAF3, disrupting the TRAF3/TRAF2/cIAP1 complex formation, thus inhibiting inflammatory signaling cascades [28]. The virulence factor EsxB is known to inhibit the activation of STING in macrophages, thereby reducing the expression of infection-induced pro-inflammatory cytokines [42]. During intracellular infection of macrophages by *S. aureus*, there was not only a suppression of pro-inflammatory cytokines but also a significant increase in the anti-inflammatory cytokine IL10 production. IL10, a major anti-inflammatory cytokine secreted by macrophages, inhibits NF-kB activation through a signal transducer and activator of transcription 3 (STAT3)-dependent pathway, thus suppressing the release of pro-inflammatory cytokines like IL1β and IL6 [43]. Furthermore, in LPS-induced inflammation, IL10 robustly inhibits mTOR phosphorylation, promoting mitophagy, which helps clear the intracellular inflammatory environment and restore mitochondrial function [22]. The literature demonstrates that pathogens can use IL10 to reduce inflammation and inhibit the antimicrobial response of immune cells. *Mycobacterium tuberculosis*, for instance, inhibits phagosome maturation in macrophages via the IL10/STAT3 pathway, facilitating its proliferation within these cells [24]. During *S. aureus* infection, the pathogen suppresses immune cell inflammatory responses via IL10, promoting the persistence of biofilm infections [44]. This study confirmed that IL10 enhances the survival of *S. aureus* within macrophages and notably reduces mtROS production, which is crucial for the pathogen's prolonged intracellular survival.

Histones rigorously regulate the transcription and translation of the IL10 gene in cells, with histone acetylation being a key epigenetic mechanism influencing gene transcription [20]. HDAC11, the sole class IV HDAC member, transfers acetyl groups to lysine residues on histone tails, relaxing chromatin structure and enhancing gene transcription [20]. Studies show that HDAC11 exerts a bidirectional regulatory function in immune modulation by targeting cytokine secretion and activating immune cells. H3K9 is a crucial target of HDAC11 deacetylation, impacting the transcription of multiple genes. Deacetylation by HDAC11 at H3K9 inhibits the transcription of cccDNA, thus constraining HBV replication [45]. Inhibition of HDAC11 upregulates OX40L in Hodgkin's lymphoma cells and reverses the immunosuppressive function of Tr1 cells [46]. Research has found that using HDAC inhibitors can upregulate the acetylation levels of H3K9, enhancing the transcription and translation of IL10 [34]. Proteomic and western blot analysis confirmed that a decrease in HDAC11 leads to increased acetylation at the H3K9 site. This reduction in HDAC11 is crucial for elevating endogenous IL10 during intracellular *S. aureus* infection, and overexpression of HDAC11 significantly inhibits IL10 transcription and translation, thus impeding *S. aureus* survival within cells. This immunoregulatory process occurs through changes in mitophagy and energy metabolism. Studies have

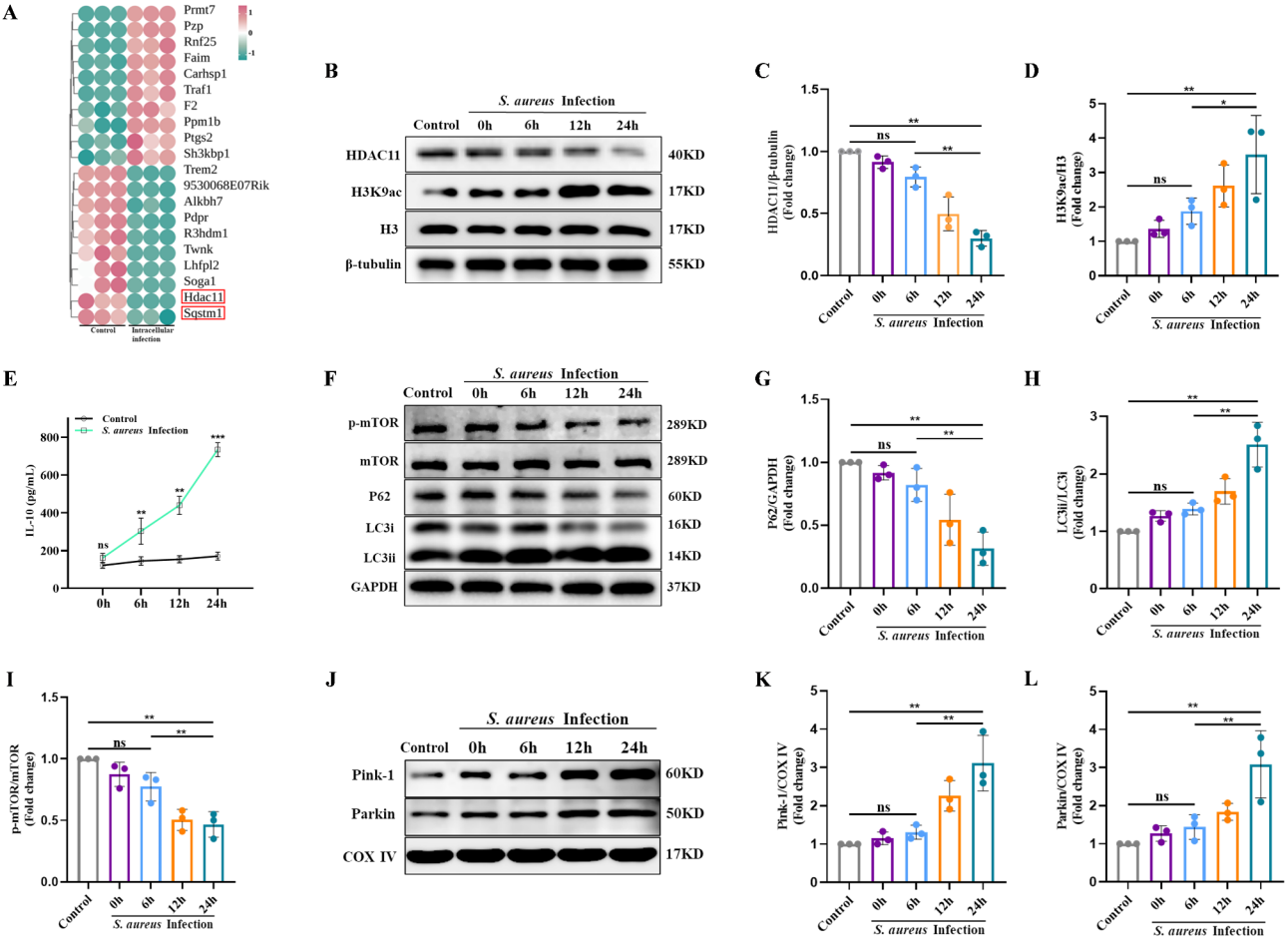


**Fig. 5** *S. aureus* infection results in the downregulation of HDAC11 in macrophages, affecting the regulation of IL10 and mitophagy. **(A)** Heatmap of the top 10 differential protein expressions increased or decreased in BMDMs 24 h after intracellular infection with *S. aureus*. **(B**, **C**, and **D)** Western blotting analysis of HDAC11 and H3K9ac protein expression levels at various times after intracellular infection of *S. aureus* (MOI = 10). β-tubulin is used as a reference protein for quantifying HDAC11 expression, and H3 for H3K9ac. **(E)** ELISA quantitative analysis of IL10 levels in the supernatant of BMDMs infected with *S. aureus* (MOI = 10) at various time points. **(F**, **G**, **H** and **I)** Western blotting analysis of LC3, P62, mTOR, and p-mTOR protein expression levels at various time points during intracellular infection. GAPDH is used as a reference protein to quantify P62 expression; changes in LC3ii/LC3i and p-mTOR/mTOR are also quantified. **(J**, **K**, and **L)** Preparation of mitochondria (Mito) and Western blotting analysis of Pink-1 and Parkin protein expression levels at various time points during intracellular infection. COX IV is used as a reference protein to quantify Pink-1 and Parkin expression levels. Statistical analysis was performed using one-way ANOVA **(C, D, G, H, I, K, L)** or two-tailed Student's unpaired t-test **(E)**: ns, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The data represent the mean ± SD from at least three independent experiments

shown that HDAC11 can mediate programmed cell death through the NLRP3/caspase-1/GSDMD pathway, and knocking out HDAC11 not only suppresses the expression of IL1β and TNF-α but also significantly inhibits programmed cell death [47]. In this study, we found that *S. aureus* intracellular infection suppresses HDAC11 expression, which may be a strategy used by *S. aureus* to avoid exposing intracellular bacteria to extracellular antimicrobial factors due to host cell death. Our findings suggest that apoptosis analysis of macrophages infected by *S. aureus* showed no significant occurrence of programmed cell death in the host cells. However, whether *S. aureus* regulates programmed necrosis through HDAC11 remains to be further investigated.

Mitochondria, crucial for energy production via oxidative phosphorylation that generates ATP, play a vital role in both cellular and organismal function. Additionally, mitochondria hold a significant position in immune functions. Research has found a close connection between mitochondria, ROS, and bacteria [39]. Mitochondria are a primary source of ROS. Upon phagocytosis of bacteria by immune cells, mitochondria can colocalize with these bacteria. Concurrently, mtROS produced by mitochondria can specifically accumulate in phagosomes containing bacteria, serving antibacterial functions [48]. Following the targeted depletion of mtROS with Mito-TEMPO, there was a notable increase in intracellular bacterial load, underscoring the critical antibacterial role of mtROS [41]. Elevated ROS levels can

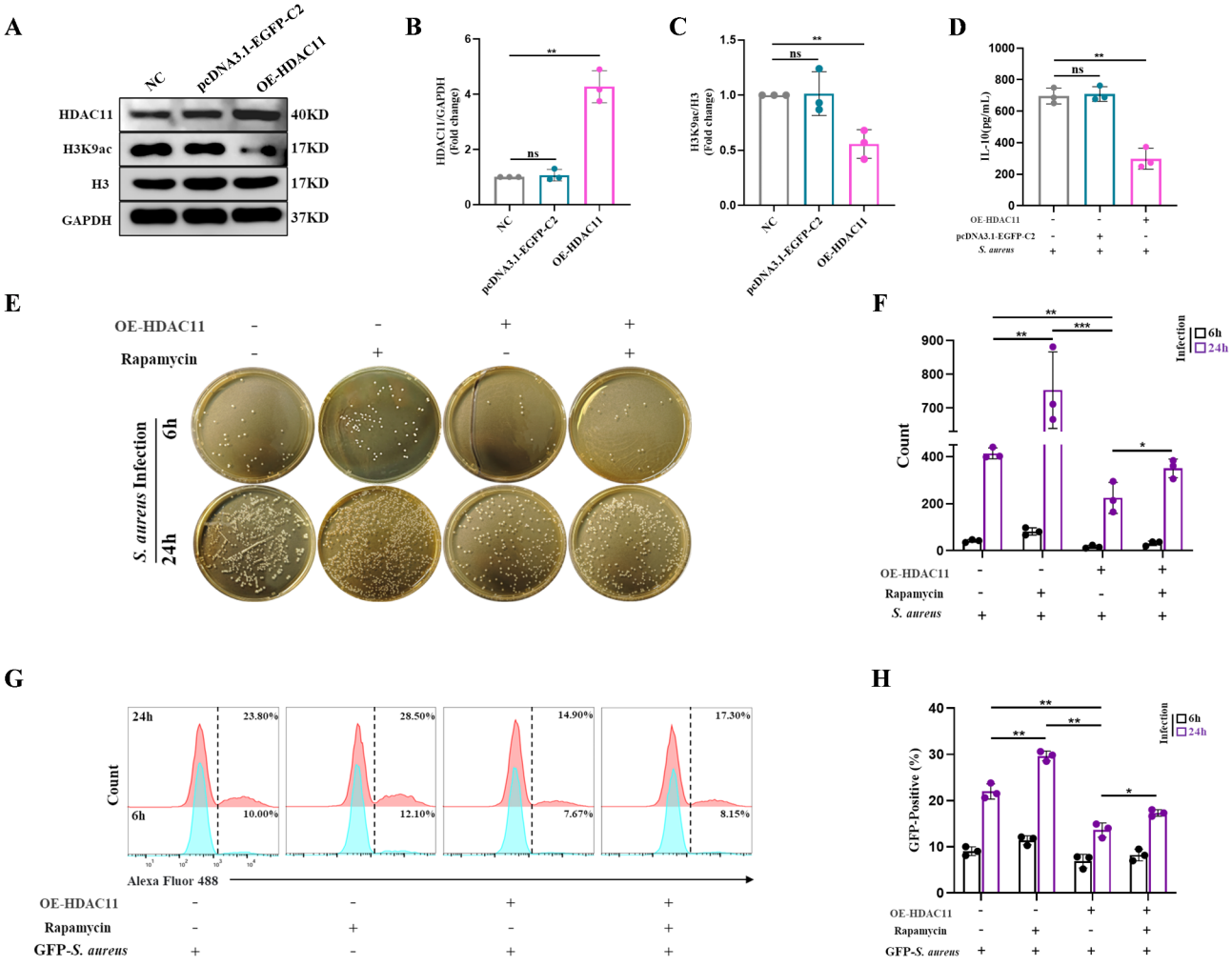


**Fig. 6** HDAC11 expression affects intracellular survival of *S. aureus* via IL10/mTOR. **(A**, **B**, and **C)** Detection of HDAC11 and H3K9ac protein levels in BMDMs overexpressing HDAC11 using Western blot. GAPDH is used as a reference protein to quantify HDAC11 expression, and H3 to quantify H3K9ac expression. **(D)** Validation of IL10 production levels induced by 24-hour intracellular *S. aureus* infection in BMDMs overexpressing HDAC11, using ELISA. **(E** and **F)** Bacterial plating and **(G** and **H)** flow cytometry to assess the impact of the mTOR inhibitor Rapamycin on intracellular bacterial load in BMDMs overexpressing HDAC11, with or without inhibitor treatment. Quantitative analysis of intracellular bacterial counts at 6 h and 24 h, and the proportion of BMDMs infected with GFP-*S. aureus*. Statistical analysis was performed using a two-tailed Student's unpaired t-test: ns, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The data are presented as the mean ± SD from at least three independent experiments

activate mitochondrial permeability transition pores, and inter-mitochondrial ROS transport can create a positive feedback loop, compromising mitochondrial membrane potential and resulting in mitochondrial swelling and structural breakdown [12]. This exacerbation of mtROS also triggers inflammasome formation, ultimately leading to cell death. Early in the intracellular *S. aureus* infection of macrophages, there was significant induction of mtROS production, which inhibited *S. aureus* growth and promoted mitochondrial membrane potential disruption, aligning with prior studies [17].

Mitophagy is an important process that mitigates mtROS production by selectively engulfing damaged mitochondria, vital for maintaining cellular homeostasis [49]. The PINK1/parkin signaling pathway is the most distinctive route involved in mitophagy. When mitochondria sustain excessive damage or are activated by oxidative stress, Drp1-mediated mitochondrial fission intensifies, segregating the damaged segments [50]. On damaged mitochondria, the loss of membrane potential inhibits PINK1 from translocating to the inner mitochondrial membrane. As a result, stabilization of PINK1 on damaged mitochondria blocks its further movement, thereby recruiting parkin. Subsequently, parkin facilitates the assembly of ubiquitin chains on proteins of the mitochondrial outer membrane. These ubiquitin chains engage autophagy cargo receptors like OPTN and P62, cooperating with the general autophagy apparatus (LC3) to encase damaged mitochondria [51]. Extensive evidence shows that bacteria can disrupt mitochondrial

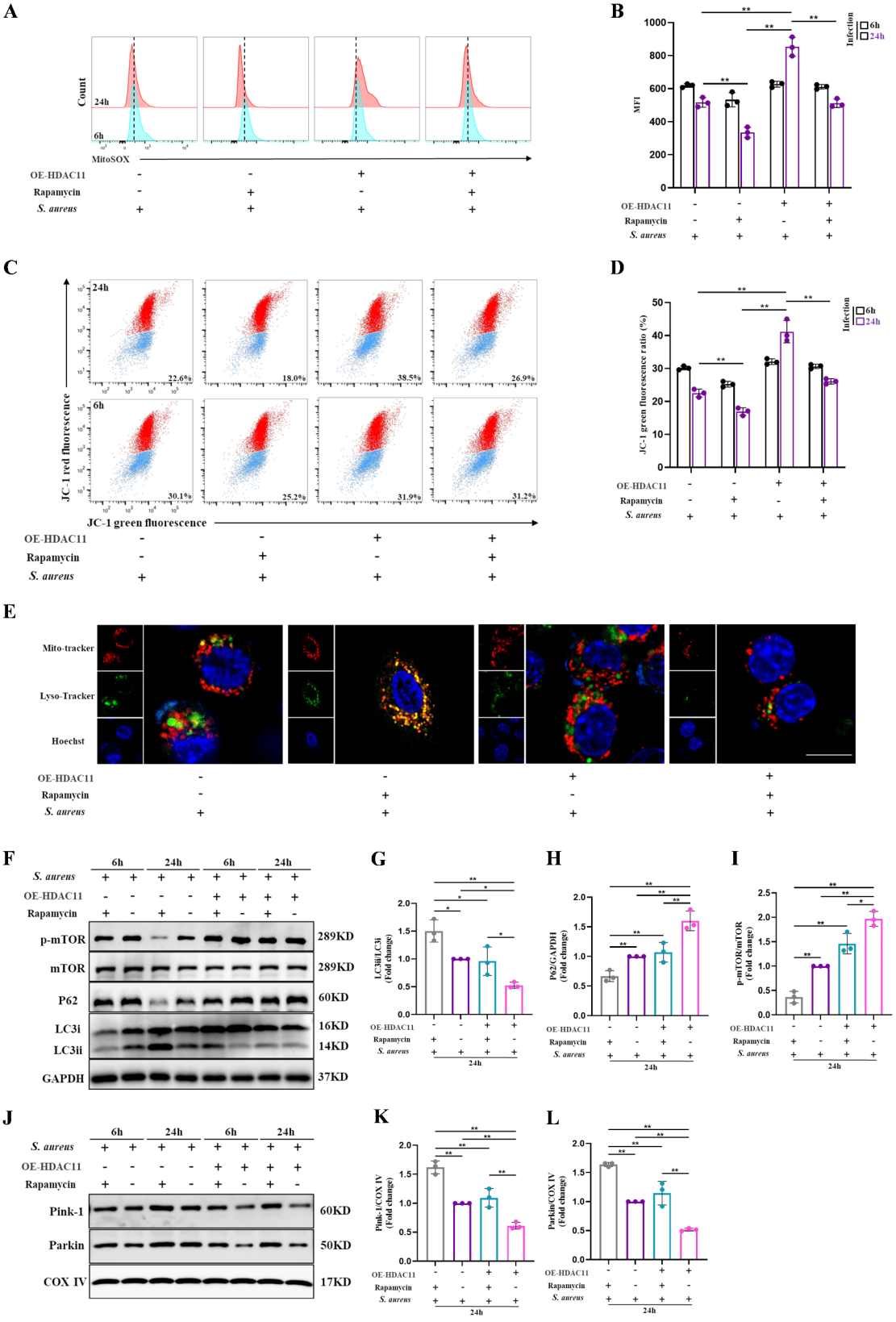


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**Fig. 7** HDAC11 affects mitophagy and intracellular mtROS clearance via mTOR. BMDMs were infected with *S. aureus* (MOI = 10) for 6 and 24 h, in conditions with or without overexpression of HDAC11 and treatment with Rapamycin (100nM). **(A** and **B)** JC-1 staining and flow cytometry analysis were used to assess changes in mitochondrial membrane potential. Quantitative analysis of the percentage of JC-1 monomer-positive cells. **(C** and **D)** Flow cytometry analysis of mtROS levels in BMDMs infected with *S. aureus* (MOI = 10) at various time points. Quantitative analysis based on Mean Fluorescence Intensity (MFI) indicating intracellular mtROS levels. **(E)** Confocal images show mitochondria (Mito-tracker, red), lysosomes (Lyso-tracker, green), and nuclei (Hochst, blue) within BMDMs (Scale bar: 10 μm). **(F**, **G**, **H**, and **I)** Western blot analysis of LC3, P62, mTOR, and p-mTOR protein expression levels in each group. GAPDH used as a reference protein for quantitative analysis of P62 expression levels and changes in LC3ii/LC3i, p-mTOR/mTOR ratios. **(J**, **K**, **and L)** Mitochondrial preparations were analyzed using western blotting to assess Pink-1 and Parkin protein expression levels in each group. COX IV was used as a reference protein to quantify the relative expression levels of Pink-1 and Parkin. Statistical analysis was performed using a two-tailed Student's unpaired t-test: ns, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The data represent the mean ± SD from at least three independent experiments

homeostasis and trigger mitophagy to suppress host inflammatory responses: Listeria monocytogenes, for example, reduces mtROS and prevents cell death by inducing mitophagy through the release of its virulence factor LLO.28 [16]. Burkholderia pseudomallei manipulates host mitophagy through its type III secretion system effector, BipD, enabling its survival within host cells [41]. Following intracellular infection by *S. aureus*, we observed that macrophages significantly produce mtROS as a defense against the pathogen. Subsequently, mitophagy is significantly activated, effectively clearing mtROS by engulfing damaged mitochondria, thereby moderating its bactericidal impact. By inhibiting mitophagy with Mdivi-1, we observed a significant increase in mtROS, which subsequently inhibited the proliferation and survival of intracellular bacteria. Mitophagy is regulated by mTOR, which can inhibit lysosome biogenesis and Drp1 phosphorylation, thereby suppressing mitophagy [35, 52]. IL10 stimulates mTOR, enhancing mitophagy to clear dysfunctional mitochondria with low membrane potential and high reactive oxygen levels, thus mitigating LPS-induced inflammation [22]. Post-Mdivi-1 treatment, supplementing with IL10 significantly curbed the rise in mtROS, suggesting that IL10 facilitates mitophagy and mtROS clearance, aiding *S. aureus* survival within cells. Further overexpression of HDAC11 revealed its critical role in initiating IL10-promoted mitophagy. Increased HDAC11 levels reduce IL10 transcription and translation, with a consequent rise in mtROS inhibiting *S. aureus* proliferation and survival.

Although it is recognized that *S. aureus* can inhibit HDAC11 to enhance IL10 expression and promote its survival, the specific identities and mechanisms of these virulence factors still require further investigation to fully elucidate the molecular mechanisms underlying *S. aureus* pathogenicity. Studies have shown that bacterial pathogens can hijack metabolic reactions in macrophages, such as Salmonella Typhimurium enhancing glycolysis and inhibiting serine synthesis to promote its intracellular survival [53]. Although our study proposes a mechanism by which *S. aureus* counteracts intracellular ROS, whether *S. aureus* interferes with mitochondrial metabolic activity and its metabolites remains an area worthy of further exploration. The simultaneous increase in the anti-inflammatory factor IL10 and pro-inflammatory cytokines during *S. aureus* intracellular infection suggests that *S. aureus* may induce an immunoregulatory state in macrophages resembling M2b macrophages [54]. This phenotypic shift is closely associated with host-pathogen interactions but cannot be fully explained within the traditional M1/M2 classification framework and requires further investigation [55].

In conclusion, these findings establish that *S. aureus* inhibits HDAC11 and utilizes the anti-inflammatory cytokine IL10 to counteract mtROS-mediated cytotoxicity in host cells. The HDAC11/IL10 pathway is essential for inducing mitochondrial autophagy, crucial for supporting *S. aureus* survival within macrophages. Our discoveries offer new insights into developing diagnostic and therapeutic strategies for recurrent osteomyelitis caused by *S. aureus* infections. This may aid in enhancing current treatment approaches and reducing recurrence rates.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-025-06161-7.

Supplementary Material 1

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**Author contributions**
Yaji Yang: Writing– original draft, Conceptualization, Methodology, Investigation, Formal analysis. Haotian Zhou: Writing– original draft, Methodology, Investigation, Formal analysis. Feilong Li: Validation, Data curation. Yanhao Zhang: Validation, Data curation. Jianye Yang: Validation, Data curation. Yidong Shen: Methodology, Investigation. Ning Hu: Methodology, Supervision, Validation. Quanming Zou: Writing– review & editing, Supervision. Leilei Qin: Writing– review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. Hao Zeng: Writing– review & editing, Supervision, Project administration. Wei Huang: Writing– review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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**Data availability**
Data will be made available on request.

## Declarations

### Ethical approval
All animal procedures were carried out in compliance with the International Guide for the Care and Use of Laboratory Animals and were authorized by the Research Ethics Committee of the Third Military Medical University (Chongqing Permit No. 2011-04). All surgical interventions were conducted under sodium pentobarbital anesthesia, with stringent measures taken to minimize suffering.

### Consent for publication
Not applicable.

### Competing interests
The authors declare that they have no competing interests.

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