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Annexin-A1 short peptide alleviates septic myocardial injury by upregulating SIRT3 and inhibiting myocardial cell apoptosis

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Abstract

Septic myocardial injury is a common complication of severe sepsis, which occurs in about 50% of cases. Patients with this disease may experience varying degrees of myocardial damage. Annexin-A1 short peptide (ANXA1sp), with a molecular structure of Ac-Gln-Ala-Tyr, has been reported to exert an organ protective effect in the perioperative period by modulating sirtuin-3 (SIRT3). Whether it possesses protective activity against sepsis-induced cardiomyopathy is worthy of study. This study aimed to investigate whether ANXA1sp exerts its anti-apoptotic effect in septic myocardial injury *in vitro* and *in vivo* via regulating SIRT3. In this study, we established *in vivo* and *in vivo* models of septic myocardial injury based on C57BL/6 mice and primary cardiomyocytes by lipopolysaccharide (LPS) induction. Results showed that ANXA1sp pretreatment enhanced the seven-day survival rate, improved left ventricular ejection fraction (EF), left ventricular fractional shortening (FS), and cardiac output (CO), and reduced the levels of creatine kinase-MB (CK-MB), cardiac troponin I (cTnI), and lactate dehydrogenase (LDH). Western blotting results revealed that ANXA1sp significantly increased the expression of SIRT3, Bcl-2, and downregulated Bax expression. TUNEL staining and flow cytometry results showed that ANXA1sp could attenuate the apoptosis rate of cardiomyocytes, whereas this anti-apoptotic effect was significantly attenuated after SIRT3 knockout. To sum up, ANXA1sp can alleviate LPS-induced myocardial injury by reducing myocardial apoptosis via SIRT3 upregulation.

Keywords: septic myocardial injury, sirtuin-3, Annexin-A1 short peptide, apoptosis

Introduction

Sepsis, one of the most common critical diseases in the intensive care unit, is often accompanied by myocardial injury caused by infection (Seymour *et al.*, 2016). Septic myocardial injury can cause severe arrhythmias, cardiac insufficiency, cardiac shock, and other complications. Data showed that patients with sepsis accompanied by myocardial injury had a poor prognosis and high mortality (Beesley *et al.*, 2018). Sepsis-induced myocardial injury is a non-ischemic disease that occurs in patients with sepsis. The main manifestations are left ventricular dilatation, decreased ventricular contractility, right ventricular dysfunction, or left ventricular (systolic or diastolic) dysfunction. The pathophysiology showed that the interstitium of cardiomyocytes was dominated by monocytes or macrophages, myocardial interstitial edema, increased interstitial collagen content, cytoplasmic vacuolization of cardiomyocytes, mitochondrial damage, and microvascular dysfunction (Hollenberg and Singer, 2021). At present, there is no effective treatment except antibiotic therapy, so it is particularly important to search for possible therapeutic methods and explore possible mechanisms from the pathophysiological and cellular levels.

Mitochondria provide energy for the various life activities of the cell and are major cellular organelles present in most cells to produce energy and perform aerobic respiration. Numerous studies have shown that the regulation of mitochondrial biosynthesis, mitochondrial autophagy, and mitochondrial dynamics play a huge role in organ protection (Yu *et al.*, 2020; Tang *et al.*, 2021). Myocardial mitochondrial dysfunction is a key factor in sepsis (Wu *et al.*, 2019). Like inflammatory responses, oxidative stress, abnormal energy metabolism, and intracellular calcium homeostasis, myocardial mitochondrial damage induces activation of apoptotic signaling pathways and induces apoptosis in cardiac myocytes (Zhang *et al.*, 2017c). In lipopolysaccharide (LPS)-treated cardiomyocytes, the prolonged opening of mitochondrial membrane permeability conversion pores and phospholipid peroxidation of mitochondrial lipid cores induced by oxidative stress can lead to cytochrome C release and activation of apoptotic pathways (Szeto, 2014). The Bcl-2 protein family, compromised of both anti-apoptotic and pro-apoptotic members, is an important mitochondrial regulator during cardiomyocyte apoptosis (Wang *et al.*, 2013). For example, rosuvastatin preconditioning reduces isoproterenol-induced myocardial injury by modulating the Bcl-2/Bax ratio (Sultan *et al.*, 2022). Irbesartan regulates Bcl-2 and Bax expression to inhibit apoptosis in myocardial ischemia-reperfusion injury (Ren *et al.*, 2019). Furthermore, Bcl-2 overexpression inhibits the innate apoptosis in rats with septic cardiomyopathy, preventing septic myocardial dysfunction (Lancel *et al.*, 2005). Thus, restoration of the balance between Bcl-2 and Bax might protect against sepsis-induced myocardial apoptosis.

Annexin-A1 (ANXA1), a Ca2+-regulated phospholipid-binding protein widely expressed in human tissues and cells(Purvis *et al.*, 2019), is involved in physiopathological processes, including growth, development, inflammation, swelling, and neurodegenerative disease (Leslie, 2015, Chua *et al.*, 2022). Also, ANXA1 can protect against cardiovascular diseases and myocardial injury (de Jong *et al.*, 2017, Ansari *et al.*, 2018). Reports state that small peptides derived from ANXA1 retain most of the effects of the full-length ANXA1 protein (Cardin *et al.*, 2017; Yu *et al.*, 2023). Since these peptides were constructed with an acetyl-blocked N-terminus, they exhibit stability and delayed proteolytic degradation (Gavins and Hickey, 2012). Annexin-A1 short peptide (ANXA1sp), a specific small tripeptide fragment of human ANXA1 developed by Dr. Zhang (Zhang *et al.*, 2010),

exhibits similar biological effects to those of ANXA1 (Sheikh and Solito, 2018). ANXA1sp has been proven to have a strong cardioprotective protective effect in the perioperative period (Zhang, Ma*, et al.*, 2017a). Furthermore, AC2-26, an ANXA1 mimetic peptide, exerts cardioprotective effects against sepsis-induced cardiomyocyte apoptosis (Zhang *et al.*, 2018). Therefore, ANXA1sp might also exert anti-apoptotic effects on cardiomyocyte apoptosis in septic myocardial injury.

Sirtuins are a family of NAD(+)-dependent deacetylases that regulate various physiological functions, from energy metabolism to stress responses (Dai *et al.*, 2018). Accumulating evidence has proven that sirtuin-mediated inhibition of the NLRP3 inflammasome exerts protective effects on myocardial cells (Sun *et al.*, 2020; Song *et al.*, 2021; Yu *et al.*, 2021). As a member of the sirtuin family, sirtuin-3 (SIRT3) can enhance the overall efficacy of the mitochondrial electron transport chain, preventing ROS production by deacetylating complexes I and III (Marcus and Andrabi, 2018). A previous report demonstrated that ANXA1sp exerts cardioprotective effects against exsanguinating cardiac arrest via SIRT3 activation (Ma *et al.*, 2019). In addition, ANXA1sp-mediated SIRT3 upregulation plays an anti-apoptotic role in ischemic kidney injury by improving mitochondrial function (Suliman *et al.*, 2021). However, whether ANXA1sp can reduce cardiomyocyte apoptosis in sepsis-induced myocardial injury by regulating the mitochondrial apoptotic pathway via SIRT3 has not yet been studied. Here, we attempted to elucidate the anti-apoptotic effects of ANXA1sp in septic myocardial injury and its association with SIRT3, thus providing new strategies for the treatment of septic myocardial injury in the future.

Materials and methods

Reagents

ANXA1sp (Molecular Mass = 445.47 Da) synthesized by GenScript (Piscataway, USA) (Zhang, Ma*, et al.*, 2017b) and LPS purchased from Sigma Aldrich were dissolved in 100% DMSO to prepare stock solutions. For the *in vivo* study, the stock solution of ANXA1sp was diluted in normal saline to a final concentration of 1 mg/ml; the stock solution of LPS was diluted in normal saline to a final concentration of 10 mg/ml. For the *in vitro* study, the stock solution of ANXA1sp was diluted in culture medium to a final concentration of 30 μM; the stock solution of LPS was diluted in culture medium to a final concentration of 1 μg/mL.

Animal study

SIRT3-KO male C57BL/6 mice (25-30 g, 8-10 weeks old) were purchased from Shanghai Model Organisms Center, Inc. Normal male C57BL/6 mice (25–30 g, 8-10 weeks old) were purchased from Changsha Tianqin Biotechnology Co., LTD. All C57BL/6 mice were maintained at Zunyi Medical University.

Normal C57BL/6 mice were assigned to four groups: Control (n=20); LPS (n=20); LPS+NS (normal saline; n=10), and LPS+ANA (ANXA1sp; n=20). SIRT3-KO C57BL/6 mice were assigned to the LPS+SIRT3-KO (n=10) or the LPS+SIRT3-KO+ANA group (n=10). For LPS induction, each mouse was intraperitoneally injected with LPS (10 mg/kg). For ANXA1sp treatment, ANXA1sp was intraperitoneally injected (1 mg/kg) once a day for three days before LPS injection. These mice were kept and monitored for lethality every 24 h for seven days. The animal study was approved by the Experimental Animal and Use Ethics Committee of Zunyi Medical University (Approval No: (2020) 2-145).

Echocardiography

Cardiac functions of mice from each group were examined by echocardiography 24 h after LPS induction. Mice were anesthetized with 1.5% isoflurane inhalation. Echocardiography was performed with a high-frequency echocardiography imaging system (Vevo2100, Canada). The left ventricular end□diastolic dimension (LVEDD), left ventricular end□systolic dimension (LVEDS), left ventricular end□diastolic volume (LVEDV), left ventricular end□systolic volume (LVESV), and cardiac output (CO) were detected. The left ventricular ejection fraction (EF) and left ventricular fractional shortening (FS) were calculated as follows: EF = LVEDV − LVESV/LVEDV × 100; FS = LVEDD − LVESD/LVEDD × 100. After echocardiographic assessment, all mice were sacrificed to collect serum samples and heart tissues for biochemical analysis and TUNEL staining.

Biochemical analysis

Serum samples were carefully collected from each group and stored at −80°C for subsequent analyses. Then, serum levels of lactate dehydrogenase (LDH), creatine kinase-MB (CK-MB), and cardiac troponin I (cTnI) were detected using corresponding commercial kits according to the manufacturer's instructions.

TUNEL staining

Briefly, heart tissues were fixed in formaldehyde, embedded in paraffin, and sectioned into 5 μm slices. Next, heart tissue sections were heated in an oven at 60□ for 15 min, dewaxed in xylene twice, and hydrated with ethanol. After digestion with proteinase K at 37□ for 30 min, each section was washed three times with PBS and incubated with TUNEL staining solution (100 μl) at 37□ for 1h. Finally, the sections were washed three times with PBS and blocked with a DAPI anti-fluorescence quencher.

Isolation of primary cardiomyocytes

Primary cardiomyocytes were isolated from SIRT3-KO and normal C57BL/6 mice as previously described (Ackers-Johnson *et al.*, 2016). Briefly, SIRT3-KO and normal C57BL/6 mice were sacrificed to remove hearts. Next, the left ventricle of the heart was digested with EDTA buffer, perfusion buffer, and collagenase buffer. After digestion, the left ventricle was diced with forceps and mechanically dissociated by repeated pipetting. Then, the cell suspension was filtered through a strainer (100 μm) and settled by gravity for 20 minutes to collect cardiomyocytes.

Primary cardiomyocyte culture and treatment

The primary cardiomyocytes isolated from SIRT3-KO and normal C57BL/6 mice were suspended in DMEM supplemented with 10% FBS and cultured in a humified atmosphere (37°C; 5% CO2) until reaching 75% confluence. Cardiomyocytes in early passages (up to passage 3) were used for further experiments.

Cardiomyocytes isolated from normal C57BL/6 mice were assigned to the Control, LPS, and LPS+ANA groups. Cardiomyocytes isolated from SIRT3-KO C57BL/6 mice were assigned to the LPS+SIRT3-KO and LPS+SIRT3-KO+ANA groups. To imitate sepsis *in vitro*, cardiomyocytes were exposed to LPS (1 μg/mL) for 12h (Mou *et al.*, 2021). For ANXA1sp treatment, cardiomyocytes were pretreated with ANXA1sp (30 μM) for 2 h before LPS induction.

Flow cytometry

The apoptosis rate of cardiomyocytes was detected by flow cytometry. The cell suspension was centrifuged at 500 ×g for 5 min in each group. The supernatant was removed and the cell precipitate was suspended with 1 ml of 1×Annexin V Binding Buffer precooled at 4□. Next, 500 μL of cell suspension was placed into a flow cytometry tube, and 5 μL of FITC-Annexin V was added and incubated at 4□ in the dark for 10 min. Then, 5 μL of Propidium Iodide was added and mixed for 5 min. The apoptosis rate was detected by a flow cytometer (Gallios, Beckman Coulter, USA).

Western blotting

The Bicinchoninic acid (BCA) method was used to detect protein concentration according to the manufacturer's instructions. Protein was transferred to a PVDF membrane and blocked with 5% skim milk at room temperature for 2 h, followed by incubation with anti-SIRT3 (cat. no. ab217319; dilution, 1:1,000; Abcam), anti-Bax (cat. no. #5023; dilution, 1:1000; Cell Signaling Technology), anti-Bcl-2 (cat. no. ab32124; dilution, 1:2000; Abcam), and anti-β-actin (cat. no. #3700; dilution, 1:1,000; Cell Signaling Technology) antibodies overnight at 4°C. Next, it was incubated with goat anti-rabbit (cat. no. #98164; dilution, 1: 5,000; Cell Signaling Technology) secondary antibodies at room temperature for 2 h. Enhanced chemiluminescence was used to detect the results. The band was imaged with the Bio-rad Imaging System.

Statistical analysis

The results in our study are shown as mean ± standard error of the mean (SEM) and were analyzed by SPSS 19.0 software. The comparison between multiple groups was analyzed by one-way analysis of variance and Tukey's post-hoc test. The survival rate of mice was assessed using the Kaplan-Meier curve. $P < 0.05$ was considered statistically significant.

RESULTS

1. ANXA1sp improved cardiac function and myocardial injury markers in septic mice.

To observe the effects of ANXA1sp on cardiac function and markers of myocardial injury in septic mice, mice were administered ANXA1sp and then subjected to LPS treatment. As illustrated by the Kaplan-Meier curve, ANXA1sp pretreatment significantly reversed the LPS-induced increase in the mortality rate of mice (Fig. 1A). In addition, the cardiac function of mice was detected by echocardiography (Fig. 1B; Table 1). It was shown that ANXA1sp partly abated the LPS-induced decline in EF, FS, and CO levels (Fig. 1C-E). Also, ELISA results revealed that the LPS-induced increase in myocardial injury markers (LDH, CK-MB, and cTnI) was also eliminated by ANXA1sp (Fig. 1F-H). In addition, ANXA1sp also reversed the LPS-mediated increase in the levels of pro-inflammatory cytokines (TNA-α, IL-1β, and IL-6) in myocardial tissues (Fig. 2A-C). Therefore, ANXA1sp improved sepsis-induced cardiac dysfunction and myocardial injury in mice.

2. ANXA1sp attenuated cardiomyocyte apoptosis *in vivo* and upregulated SIRT3 expression.

To observe the effect of ANXA1sp on apoptosis, TUNEL staining was used to calculate the apoptosis rate. As shown in Fig. 3A-B, LPS caused cardiomyocyte apoptosis *in vivo*, which was abated by ANXA1sp pretreatment. Similarly, ANXA1sp pretreatment reversed the LPS-induced increase in Bax levels and decrease in Bcl-2 levels. (Fig. 3C-E). In addition, LPS treatment reduced SIRT3 expression in cardiac tissues, which was revoked by ANXA1sp administration (Fig. 3C and

F). Taken together, ANXA1sp might reduce LPS-induced cardiomyocyte apoptosis *in vivo* by upregulating SIRT3 expression.

3. ANXA1sp exerted myocardial protective effects through SIRT3

Previous experiments have shown that SIRT3 plays an important role in cardiac protection. Therefore, we further investigated the role of SIRT3 in the protective effect of ANXA1sp against LPS-induced cardiac dysfunction and myocardial injury. After SIRT3 knockout, mice were also administered ANXA1sp and subjected to LPS treatment. ANXA1sp failed to protect against LPS-induced cardiac dysfunction (Fig. 4A-D; Table 2) and myocardial injury (Fig. 3E-G) in SIRT3-knockout mice. Besides, ANXA1sp hardly blocked the LPS-mediated increase in TNA-α, IL-1β, and IL-6 levels in myocardial tissues from SIRT3-knockout mice (Fig. 5A-C). Therefore, ANXA1sp might exert protective effects against cardiac dysfunction and myocardial injury in LPS-challenged mice by regulating SIRT3 expression.

4. ANXA1sp attenuated cardiomyocyte apoptosis by upregulating SIRT3

Next, the role of SIRT3 in the protective effect of ANXA1sp against LPS-induced cardiomyocyte apoptosis was examined. As shown in Fig. 6A, ANXA1sp pretreatment failed to reverse the LPS-induced increase in the mortality rate of SIRT3-knockout mice. In addition, upon SIRT3 knockout, ANXA1sp could not reduce the LPS-induced cardiomyocyte apoptosis rate *in vivo* (Fig. 6B and C). Also, ANXA1sp failed to reverse LPS-induced changes in Bax and Bcl-2 levels or upregulate SIRT3 expression in cardiac tissues from SIRT3-knockout mice (Fig. 6D-G). To sum up, ANXA1sp reduced cardiomyocyte apoptosis by upregulating SIRT3 expression in LPS-induced mice.

5. ANXA1sp inhibited cardiomyocyte apoptosis by up-regulating SIRT3 *in vitro*.

To verify the cardioprotective effect of ANXA1sp *in vitro*, primary cardiomyocytes were extracted from the heart tissues of mice from each group and then subcultured. Cardiomyocytes were pretreated with ANXA1sp for 2 h and LPS for 12h. The apoptosis rate of cardiomyocytes was detected by flow cytometry. ANXA1sp pretreatment can reduce the LPS-induced apoptosis of cardiomyocytes; meanwhile, ANXA1sp failed to diminish the apoptosis rate of cardiomyocytes upon SIRT3 knockout (Fig. 7A-F). Therefore, ANXA1sp inhibited LPS-induced cardiomyocyte apoptosis by upregulating SIRT3 *in vitro*.

Discussion

In this study, we showed that (1) ANXA1sp improved myocardial injury in sepsis. (2) ANXA1sp upregulated SIRT3 and Bcl-2 expression and upregulated Bax expression, producing an anti-apoptotic effect. (3) Upon SIRT3 knockout, the anti-apoptotic effect of ANXA1sp was obviously weakened, indicating that SIRT3 was an anti-apoptotic target and ANXA1sp could reduce myocardial injury in sepsis by improving cardiomyocyte apoptosis through the SIRT3 target.

Current research on septic myocardial injury contains both clinical and animal studies; however, it is very difficult to find effective drugs to treat myocardial injury in sepsis in the clinical setting (Meng *et al.*, 2016). Therefore, attention has been turned to the exploration of mechanisms in animal models with the aim of finding possible drugs to address this challenge. Accumulating evidence suggests that drugs produce protective effects against septic myocardial injury by regulating apoptosis-related signaling pathways (Xu *et al.*, 2020a; Zhou *et al.*, 2020), suggesting that anti-

apoptotic drugs might be promising candidates to block the development of septic myocardial injury. Consistent with previous findings, our study revealed that ANXA1sp enhanced the seven-day survival rate of LPS-challenged mice and protected against cardiac dysfunction and myocardial apoptosis by upregulating Bcl-2 expression and downregulating Bax expression.

Studies have demonstrated that the sirtuin family can exert antisepsis effects through the deacetylation of inflammation-related cytokines, oxidative stress-related factors, and proapoptotic factors (Sun *et al.*, 2021). As an evolutionarily conserved nicotinamide adenine dinucleotide-dependent (NAD+) histone deacetylation protein mainly localized in mitochondria, SIRT3 can maintain mitochondrial activity by regulating processes such as oxidative stress, energy metabolism, mitochondrial membrane permeability, and mitochondrial homeostasis (Ansari *et al.*, 2017). As a mitochondrial regulator involved in apoptosis regulation, SIRT3 upregulation can inhibit apoptosis and reduce cell damage. For instance, melatonin alleviates autophagy and apoptosis by upregulating SIRT3 (Wang *et al.*, 2021). Stilbene glycosides upregulate SIRT3 and inhibit neuronal apoptosis in ischemic stroke (Li *et al.*, 2021), SIRT3 protects pancreatic β cells from endoplasmic reticulum stress-induced apoptosis and dysfunction (Zhang *et al.*, 2016).

SIRT3 exerts protective effects against cardiac disorders, including septic myocardial injury (Xin & Lu, 2020). Much evidence indicates that SIRT3 is a potential target for the treatment of septic cardiomyopathy (Zheng *et al.*, 2017; Xu *et al.*, 2020b). Recent research has also proven that natural biomolecules, including Quercetin (D'Aria *et al.*, 2017), may exert potential cardioprotective roles by activating SIRT3(Chen *et al.*, 2021; Li *et al.*, 2022; Liu and Zhao, 2022). In this study, ANXA1sp reversed LPS-induced SIRT3 downregulation in normal C57BL/6 mice. After LPS induction, SIRT3-knockout mice exhibited a reduced seven-day survival rate but aggravated cardiac dysfunction and myocardial apoptosis compared with normal mice. In addition, ANXA1sp pretreatment failed to upregulate SIRT3 expression or improve the seven-day survival rate, cardiac dysfunction, and myocardial apoptosis in SIRT3-knockout mice. Taken together, ANXA1sp protected against cardiac dysfunction and myocardial apoptosis in septic cardiomyopathy by upregulating SIRT3 expression.

However, this study has some limitations. We have not observed how ANXA1sp enters the cells or how it interacts with them to play an anti-apoptotic role. In the future, we will design more reasonable experiments to clarify this process. Besides, future studies will identify additional targets for ANXA1sp in sepsis-induced myocardial injury.

All in all, in our study, LPS is used to simulate the microenvironment of cardiomyocytes in septic cardiomyopathy. LPS insult can reduce the expression of SIRT3, while ANXA1sp pretreatment can promote SIRT3 and Bcl-2 expression and inhibit Bax expression. However, upon SIRT3 knockout, the protective effect of ANXA1sp was not obvious, suggesting that ANXA1sp exerts this protective effect by targeting SIRT3. This study highlights a promising drug and a potential target for the treatment of sepsis-induced myocardial injury.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

SQ, YR, BF, JL, and XF participated in the study design and manuscript writing and confirmed the authenticity of all the raw data. HM, KL, HY, JL, QD, and XW performed the experiments, analyzed the data, and participated in manuscript writing. All authors have read and approved the final manuscript.

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Figure Legends

Figure 1. The seven-day survival rate of mice treated with LPS and the effect of ANXA1sp on cardiac function and myocardial injury markers in septic mice. (A) Mice were divided into Control (n=10), LPS (n=10), LPS+NS (n=10), and LPS+ANA (n=10) groups. The survival rate of mice was evaluated using Kaplan-Meier curves. (B) Representative left ventricular echocardiograms of mice from each group. (C-E) EF, FS, and CO of mice from each group. (F-H) ELISA for LDH, CK-MB, and cTnI in the blood of mice from each group. *NS for normal saline, ANA for ANXA1sp, n=3,* $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, *ns for no significance.*

Figure 2. ANXA1sp ameliorated myocardial inflammatory responses in septic mice. (A-C) ELISA for TNA-α, IL-1β, and IL-6 levels in myocardial tissues from each group. *NS for normal saline, ANA for ANXA1sp, n=3,* $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, *ns for no significance.*

Figure 3. ANXA1sp upregulated SIRT3 and attenuated cardiomyocyte apoptosis. (A and B) TUNEL staining for cardiomyocyte apoptosis *in vivo*. (C-F) Relative expression levels of Bcl-2, Bax, and SIRT3 in cardiac tissues by Western blotting. *NS for normal saline, ANA for ANXA1sp, n=3,* $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, *ns for no significance.*

Figure 4. ANXA1sp exerted myocardial protective effects via SIRT3. (A) Mice were divided into Control (n=10), LPS (n=10), LPS+NS (n=10), LPS+ANA (n=10), LPS+SIRT3-KO, and LPS+SIRT3-KO+ANA groups. Representative left ventricular echocardiograms of mice from each group. (B-D) EF, FS, and CO of mice from each group. (E-G) ELISA for LDH, cTnI, and CK-MB in blood samples of mice from each group. *NS for normal saline, ANA for ANXA1sp, SIRT3-KO for SIRT3 knockout, n=3, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns for no significance.*

Figure 5. ANXA1sp alleviated myocardial inflammatory responses via SIRT3. (A-C) ELISA for TNA-α, IL-1β, and IL-6 levels in myocardial tissues from each group. *NS for normal saline, ANA for ANXA1sp, SIRT3-KO for SIRT3 knockout, n=3, *P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, *ns for no significance.*

Figure 6. ANXA1sp attenuated cardiomyocyte apoptosis by upregulating SIRT3. (A) The survival rate of mice was evaluated using Kaplan-Meier curves. (B and C) TUNEL staining for cardiomyocyte apoptosis *in vivo*. (D-G) Relative protein levels of Bcl-2, Bax, and SIRT3 in heart tissues from each group by Western blotting. *NS for normal saline, ANA for ANXA1sp, SIRT3-KO for SIRT3 knockout, n=3, *P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, *ns for no significance.*

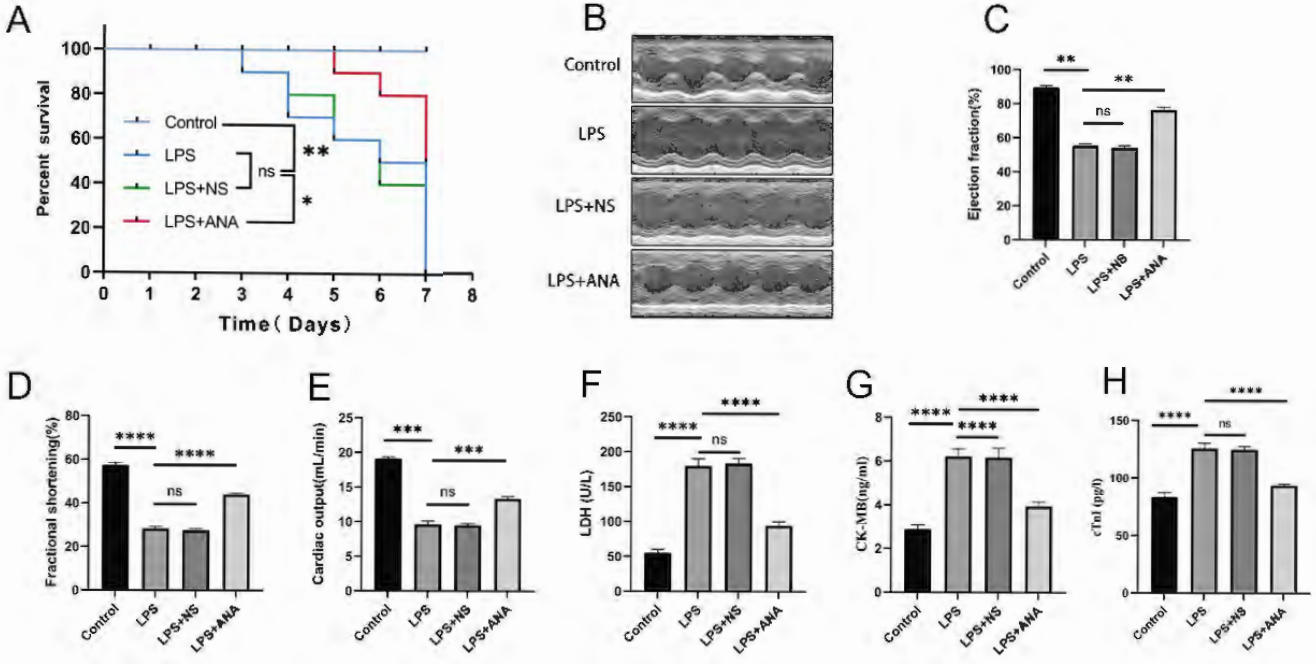
Figure 7. ANXA1sp inhibited cardiomyocyte apoptosis by upregulating SIRT3 *in vitro*. (A-F) The apoptosis of primary cardiomyocytes in each group was detected by flow cytometry. *NS for normal saline, ANA for ANXA1sp, SIRT3-KO for SIRT3 knockout, n=3, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns for no significance.*

Table 1. Effects of ANXA1sp on mouse cardiac function.

	Control	LPS	LPS+NS	LPS+ANA
LVEDD (mm)	4.22 ± 0.35	3.89 ± 0.19	4.02 ± 0.18	4.12 ± 0.09
LVEDS (mm)	2.03 ± 0.17	2.89 ± 2.15∗	2.91 ± 2.15	2.31 ± 0.10∗
LVEDV (μl)	79.85 ± 2.35	68.15 ± 1.82∗	67.3 ± 2.55	76.8 ± 1.32∗
LVESV (μl)	13.85 ± 1.56	28.91 ± 3.21∗	29.12 ± 2.71	18.2 ± 2.15∗

Table 2. SIRT3 is involved in ANXA1sp-mediated improvement of cardiac function in LPS-induced mice.

	Control	LPS	LPS+ANA	LPS+SIRT3-KO	LPS+SIRT3-KO+ANA
LVEDD (mm)	4.23 ± 0.27	3.92 ± 1.09	4.12 ± 0.12	3.99 ± 0.55	4.02 ± 0.22
LVEDS (mm)	2.09 ± 0.35	2.87 ± 2.24∗	2.31 ± 0.32∗	3.15 ± 0.11∗	3.21 ± 1.05
LVEDV (μL)	79.58 ± 1.16	67.88 ± 1.37∗	77.9 ± 1.51∗	61.5 ± 0.16∗	62.1 ± 1.81
LVESV (μL)	14.13 ± 2.37	29.12 ± 2.15∗	17.9 ± 2.22∗	35.8 ±2.32∗	34.6 ±2.27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Percent survival
Time (Days)
Control
LPS
LPS+NS
LPS+ANA
ns
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*
B
Control
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C
Ejection fraction(%)
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Fractional shortening(%)
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Cardiac output(mL/min)
F
LDH (U/L)
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CK-MB(ng/ml)
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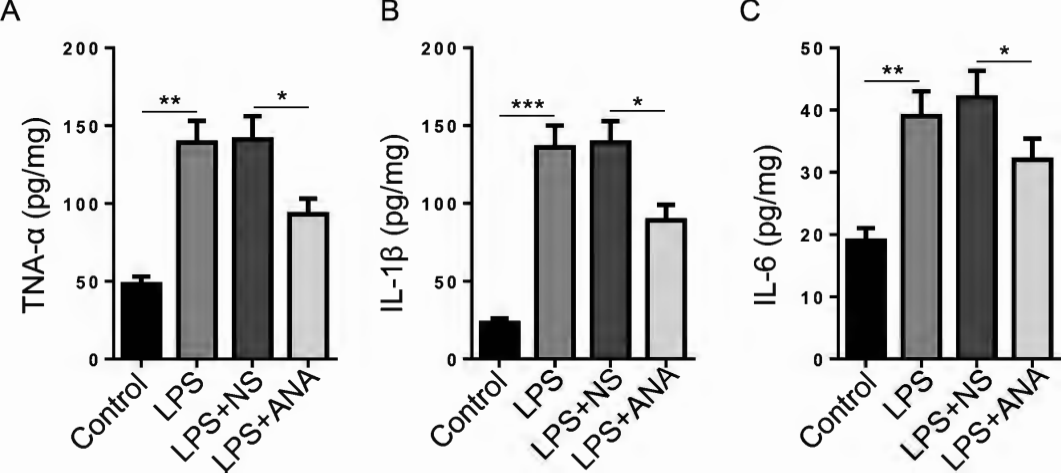
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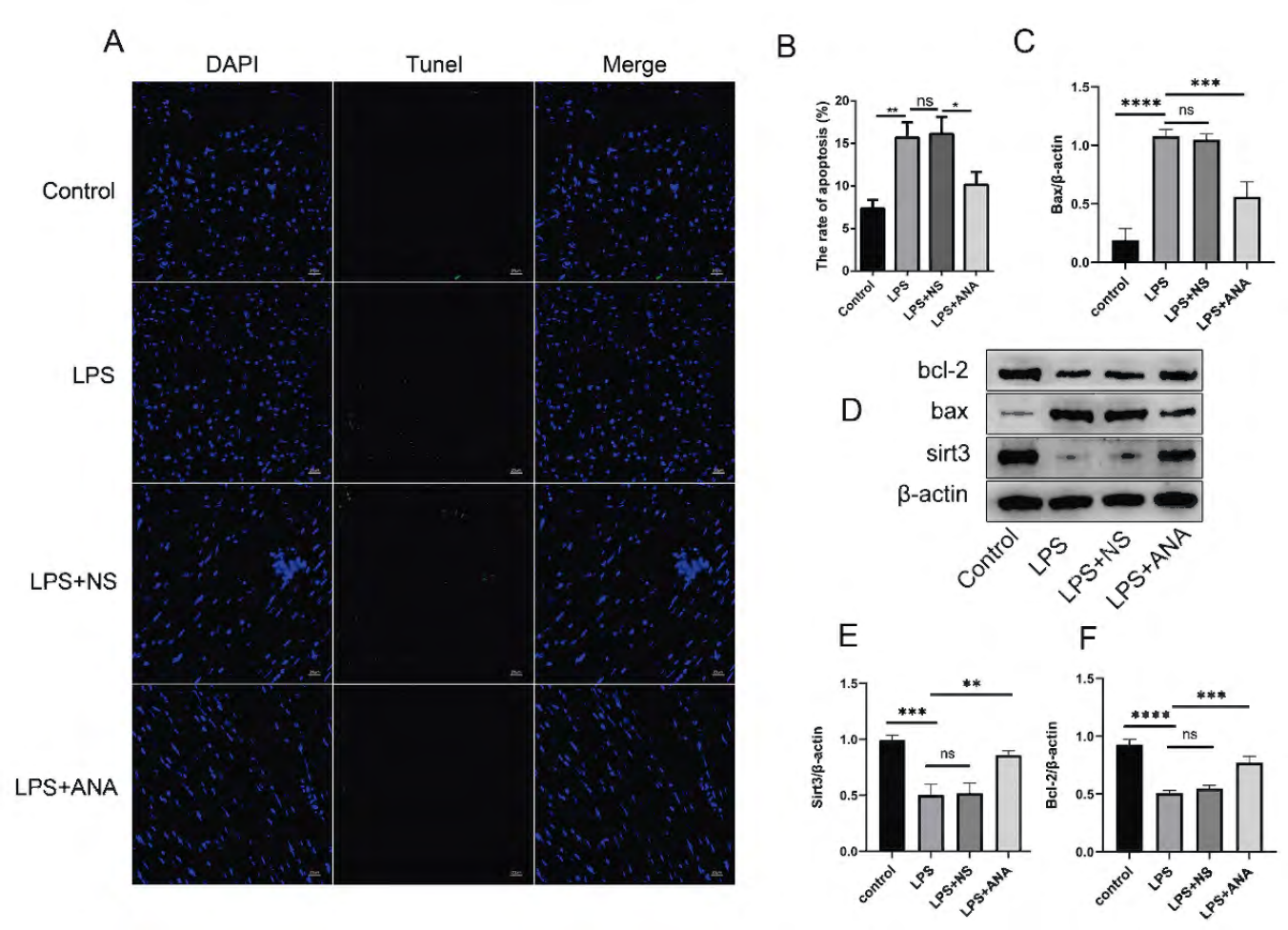
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Bax/β-actin
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β-actin
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Sirt3/β-actin
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LPS+NS
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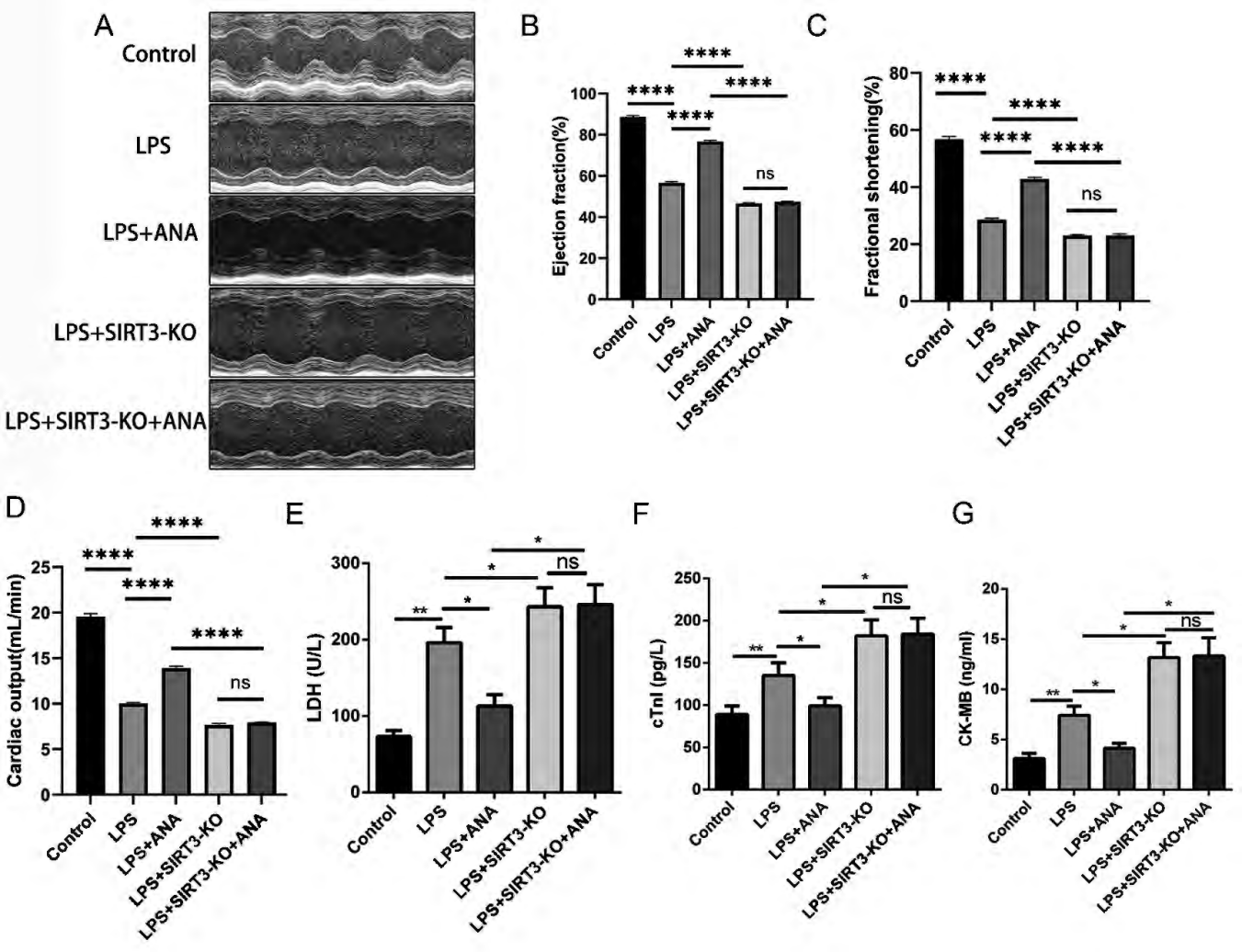
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Control
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LPS+ANA
LPS+SIRT3-KO
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Fractional shortening(%)
D
Cardiac output(mL/min)
E
LDH (U/L)
F
cTnI (pg/L)
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Control
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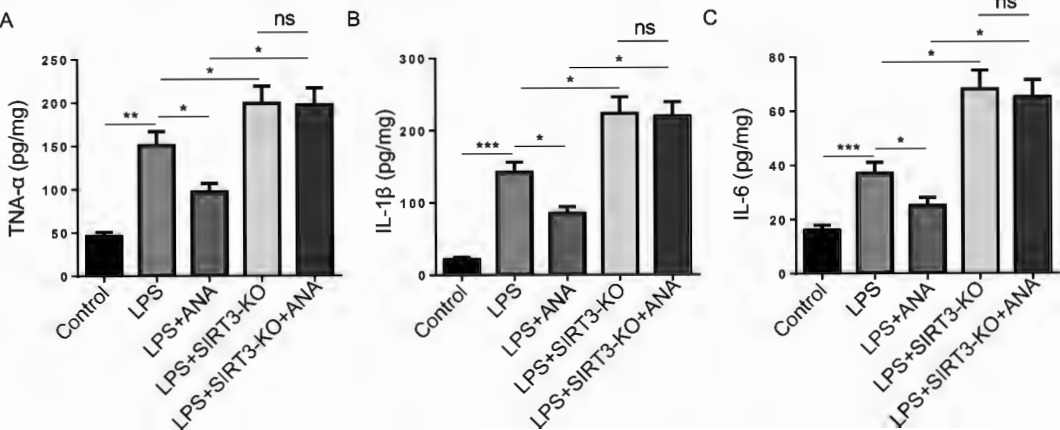
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Control
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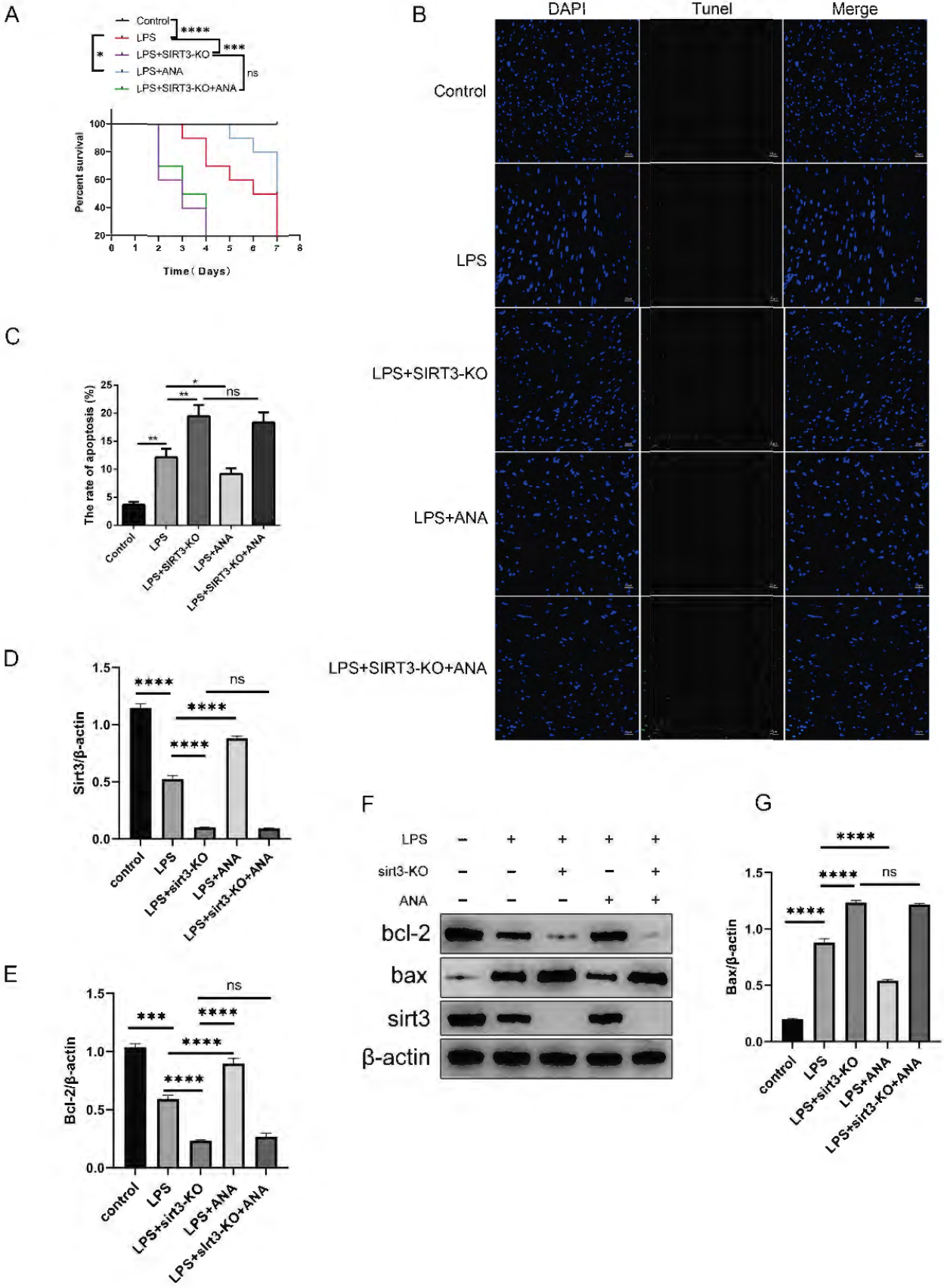
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Percent survival
Time(Days)
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DAPI
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Control
LPS
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LPS+ANA
LPS+SIRT3-KO+ANA
C
The rate of apoptosis (%)
ns
Control
LPS
LPS+SIRT3-KO
LPS+ANA
LPS+SIRT3-KO+ANA
D
Sirt3/β-actin
control
LPS
LPS+sirt3-KO
LPS+ANA
LPS+sirt3-KO+ANA
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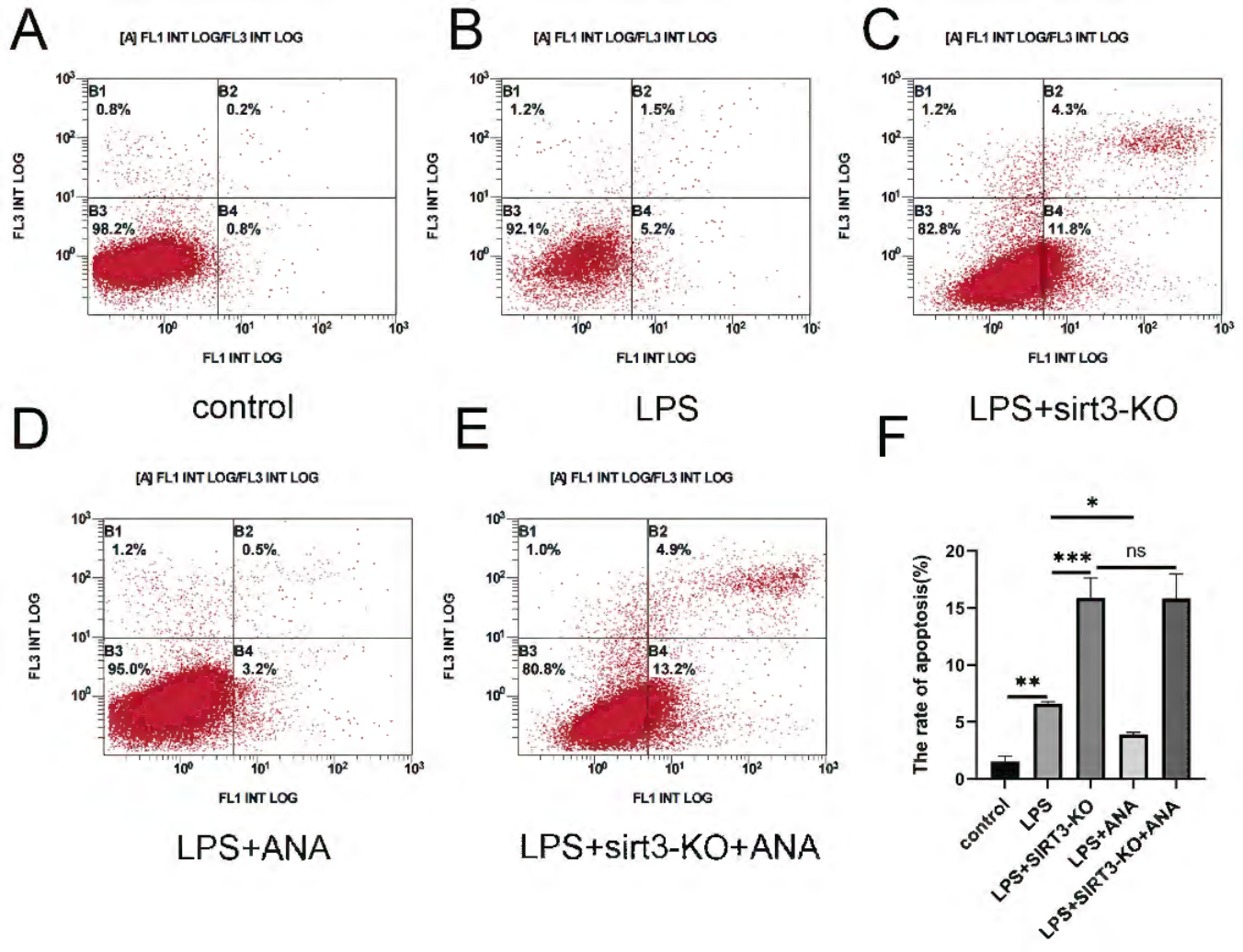
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control
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FL1 INT LOG
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FL1 INT LOG
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FL1 INT LOG
LPS+ANA
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FL3 INT LOG
FL1 INT LOG
LPS+sirt3-KO+ANA
F
The rate of apoptosis(%)
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ns
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control
LPS
LPS+SIRT3-KO
LPS+ANA
LPS+SIRT3-KO+ANA