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Original Article

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miR-155 induces sepsis-associated damage to the intestinal mucosal barrier via sirtuin 1/nuclear factor-κB-mediated intestinal pyroptosis

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Abstract

Sepsis is a life-threatening state of organ dysfunction caused by systemic inflammation and a dysfunctional response to host infections that can induce severe intestinal mucosal damage. Pyroptosis is mediated by the activated NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome after stimulation by various inflammatory factors during sepsis. The inflammatory response is a major driver of intestinal damage during sepsis. Intestinal mucosal barrier dysfunction in sepsis is associated with pyroptosis, a type of programmed inflammatory cell death. Several studies have confirmed the role of miR-155 in sepsis and other diseases. However, the effect of miR-155 on intestinal pyroptosis in the context of intestinal mucosal barrier dysfunction during sepsis remains unclear. Thus, a model of sepsis in Sprague-Dawley rats is established using cecal ligation and puncture (CLP), and a series of molecular biological methods are used in this study. The results show that the expression of miR-155 is increased and that of sirtuin 1 (SIRT1) is decreased in the intestinal tissues of patients with sepsis. miR-155 expression is negatively correlated with SIRT1 expression. Increased miR-155 expression significantly inhibits SIRT1 activity and upregulates the expressions of NOD-like receptor family pyrin domain-containing 3 (NLRP3), caspase-1, apoptosis-associated speck-like protein containing a CARD (ASC), interleukin-1β (IL-1β) and interleukin-18 (IL-18) to promote pyroptosis. The inhibition of miR-155 expression is associated with increased SIRT1 expression, promotes the deacetylation of p65, and significantly downregulates p65 acetylation. Herein, we propose that miR-155 induces pyroptosis in the intestine partly by regulating SIRT1, thereby reducing the deacetylation of the nuclear factor (NF)-κB subunit p65 and increasing NF-κB signaling activity in sepsis, leading to intestinal barrier damage.

Key words intestinal barrier dysfunction, miR-155, NF-κB, pyroptosis, sepsis, SIRT1

Introduction

Sepsis, defined as life-threatening organ dysfunction caused by systemic inflammation and a dysfunctional response to host infection [1], is one of the leading causes of death in intensive care units [2]. Previous studies have shown that intestinal mucosal barrier dysfunction in sepsis plays an important role in the occurrence and development of multiple organ dysfunction [3,4]. Under normal circumstances, the intestinal mucosal barrier prevents microorganisms and their products from entering the blood from the intestine. During sepsis, excessive inflammation damages intestinal epithelial cells, damages the intestinal mucosal barrier and promotes bacterial migration and toxin transmission. This can lead to systemic inflammatory response syndrome and multiple organ dysfunction syndrome [5]. Therefore, the intestine is considered a "motor" for sepsis and multiple organ dysfunction [6]. The integrity of the intestinal mucosal epithelial cell layer and tight junctions between epithelial cells are important factors for maintaining intestinal barrier function. Severe diseases such as sepsis cause increased intestinal permeability, starting 1 h after sepsis at the earliest and continuing for 48 h [7]. This impaired barrier function is primarily mediated by changes in tight junctions and associated proteins, mainly claudins and occludin, and allows the

lumen contents to flow out, potentially damaging distant organs; however, the mechanisms of epigenetic regulation are unclear. An increasing number of studies have shown that the breakdown of intestinal barrier function and an increase in intestinal mucosal permeability are strongly correlated with the dysregulation of intestinal epithelial death [8]. Dysregulation of intestinal epithelial cell death, including apoptosis, pyroptosis, necrosis, and ferroptosis, destroys the integrity of the intestinal barrier and allows luminal antigens to invade the lamina propria. This can lead to acute and chronic inflammatory responses.

Pyroptosis is an inflammatory programmed cell death mode involving specific proteins and is characterized by the formation of cell membrane pores, cell swelling and lysis, and the release of intracellular inflammatory contents, resulting in a rapid and robust inflammatory response [9]. The NLR family pyrin domain containing protein 3 (NLRP3) inflammasome is a widely studied pyroptosis pathway. It comprises a sensor (NLRP3), an adapter (apoptosis-associated speck-like protein containing a CARD, ASC), and an effector (caspase-1) [10]. NLRP3 interacts with ASC, which is associated with apoptosis, recruits pro-caspase-1, and cleaves it to form active caspase-1. Activated caspase-1 cleaves interleukin (IL)-1β, and IL-18 preforms. By promoting its maturation and secretion, activated caspase-1 can also activate gasdermin D (GSDMD), causing it to split the N-terminal domain, which forms a perforated channel on the cell membrane, promoting the inflow of water molecules and the outflow of inflammatory factors, inducing an acute inflammatory response and ultimately leading to cell pyroptosis [11]. Previous studies have shown that pyroptosis is involved in the occurrence and development of various inflammatory and infectious diseases [12,13]. In recent years, an increasing number of studies have suggested that pyroptosis may play an important role in sepsis and sepsis-related organ dysfunction [14,15], but the specific mechanism underlying the occurrence of intestinal mucosal barrier dysfunction in sepsis still requires further research.

MicroRNAs (miRNAs) are endogenous, noncoding, single-stranded, small-molecule RNAs composed of approximately 22–25 nucleotides [16]. They can regulate the expressions of target genes at the posttranscriptional level by degrading target genes or inhibiting their translation through complete or incomplete complementary pairing with the 3′ noncoding region of target genes [17]. Recently, the role of miRNAs in the development and occurrence of sepsis has received considerable attention. Among these miRNAs, miR-155, a common immunomodulatory miRNA, is induced by lipopolysaccharide to control inflammation in multiple cells and organs [18]. Wang *et al*. [19] reported that the expression level of miR-155 is significantly increased in lipopolysaccharide-induced sepsis mouse and macrophage models and that serum/glucocorticoid-regulated kinase family member 3 (SGK3) is a potential target of miR-155. A meta-analysis of the diagnostic value of noncoding RNAs in sepsis revealed that noncoding RNAs, especially miR-155, may be effective biomarkers for diagnosing sepsis [20]. However, the role of miR-155 in sepsis-induced dysfunction of the intestinal mucosal barrier remains unclear.

SIRT1, a member of the SIRT family, is an important deacetylase in the nucleus that migrates to the cytoplasm under specific conditions. SIRT1 regulates key metabolic processes by deacetylating various substrates (NF-κB, p53, FOXO1, *etc*.), including gene silencing, oxidative stress, aging, apoptosis, and inflammation [21,22]. SIRT1 has been widely studied for its protective role in inhibiting the inflammatory response, reducing apoptosis, protecting mitochondrial function and inhibiting oxidative stress [23]. Among them, the nuclear transcription factor NF-κB, a heterodimer protein composed of two functional subunits, p65 and p50, is the central mediator of the inflammatory response and fundamentally participates in the inflammatory response [24]. SIRT1 directly acts on p65/RelA, reduces Lys310 acetylation level, inhibits its transcriptional activity, and downregulates the expressions of downstream genes [25]. However, the involvement of SIRT1 in intestinal pyroptosis and intestinal mucosal barrier dysfunction remains unclear.

In this study, we propose that miR-155 is involved in the development of intestinal mucosal barrier dysfunction in sepsis. The regulation of miR-155 expression and its effects on intestinal dysfunction in sepsis were confirmed using our model. Our data revealed that miR-155 regulates intestinal epithelial pyroptosis by downregulating SIRT1, partly by regulating the deacetylation of the NF-κB subunit p65 and NF-κB signaling activity. This study provides a new approach for preventing and treating intestinal mucosal barrier dysfunction in sepsis patients.

Materials and Methods

Animals

Seventy-two male Sprague-Dawley rats (weighing 180–250 g, aged 8–10 weeks) were acquired from the Experimental Animal Center of Xinjiang Medical University. The rats were housed under temperature- and humidity-controlled conditions with free access to water and food. All animal experimental procedures were approved by the Animal Ethics Committee of the Animal Laboratory Center of Xinjiang Medical University (certification number: IACUC-20220728-09).

Rat model of sepsis

Based on previous literature [26], we established a sepsis model by using cecal ligation and puncture (CLP). Briefly, isoflurane was used for inhalation anesthesia. After anesthesia, a 2-cm incision was made through the midline of the abdomen, the cecum was exposed, and the end of the cecum was selected from the middle of the ileocecal flap for ligation. A 21-G needle was used to penetrate the cecum twice, the cecum was gently squeezed until a small amount of intestinal content was spilled, the cecum was returned to the abdomen, and the incision was stitched layer by layer. In the sham group, the abdominal cavity was opened using the same method, but only the cecum was observed without ligation or perforation. After the operation, each rat was subcutaneously injected with normal saline (40 mg/kg) for liquid resuscitation. Twenty-four hours after CLP, the rats were sacrificed by CO_2 inhalation, intestinal tissues were isolated, and the serum was collected.

Animal grouping and drug administration

According to the experimental requirements, the animals were randomly divided into the sham, CLP, CLP+DMSO, CLP+EX-527, CLP+SRT1720, CLP+AAV-NC, CLP+AAV-miR-155-overexpression, CLP+AAV-miR-155-inhibitor and CLP+AAV-miR-155-inhibitor+EX-527 groups, with 8 rats in each group. AAV-miR-155-overexpressing, AAV-miR-155-inhibitor and their scrambled sequence control (HANBIO, Shanghai, China) at a dose of 1×10^{12} vector genomes (v.g.) were administered via intraperitoneal injection four weeks before CLP modeling. SRT1720 hydrochloride (HY-15145; Med-

ChemExpress, Monmouth Junction, USA), a SIRT1-specific agonist, and EX-527 (HY-15452; MedChemExpress), a SIRT1 inhibitor, were administered intraperitoneally 2 h after CLP modeling (10 mg/kg).

Hematoxylin and eosin staining

The intestinal tissue was fixed in 4% paraformaldehyde for 24 h, paraffin-embedded, and then cut into 4-μm sections. Sections were stained with hematoxylin and eosin for 50 s. Histomorphological changes were observed using an optical microscope (Ni-U; Nikon, Tokyo, Japan).

Immunohistochemical (IHC) staining

Paraffin sections were heated at 60°C for 2 h and then dewaxed with xylene and an alcohol gradient. Antigen repair was performed using 1× EDTA at 100°C for 7 min. Then, the sections were washed three times with phosphate-buffered saline (PBS) (5 min/wash), after which they were blocked with goat serum for 30 min and incubated with primary antibody specific for SIRT1 (#8469, 1:300; CST, Boston, USA), claudin-1 (28674-1-AP, 1:500; Proteintech, Wuhan, China), and occludin (27260-1-AP, 1:500; Proteintech) overnight at 4°C. The next day, after three further washes, the secondary antibody was labeled with biotin and streptomycin-conjugated anti-biotin peroxidase (Proteintech) for 30 min. After washing three times with PBS, the DAB color developed for 40 s. Finally, the tablets were stained with hematoxylin, differentiated with hydrochloric alcohol, reverted blue with PBS, dehydrated with an alcohol gradient, and then sealed with neutral gum. Finally, the positioning and expression of each parameter were observed under the Ni-U optical microscope.

Immunofluorescence (IF) staining

The paraffin dewaxing and antigen retrieval processes were the same as those used for IHC. After antigen retrieval, the sections were washed three times with PBS, after which they were permeabilized for 30 min with 0.5% Triton X-100. Then, the sections were blocked with goat serum for 30 min and incubated overnight with primary antibodies specific for GSDMD (AF4012, 1:50; Affinity, Cincinnati, USA), caspase-1 (22915-1-AP, 1:50; Proteintech), ASC (DF6304, 1:50; Affinity), and NLRP3 (WL02635, 1:200; Wanleibio, Shenyang, China) at 4°C. The next day, the sections were incubated with a secondary antibody (RGAR002, 1:500; Proteintech) for 2 h and DAPI for 5 min. After being sealed with 50% glycerin, the sections were observed under a confocal laser scanning fluorescence microscope (AXR+NSPARC; Nikon).

Transmission electron microscopy (TEM)

The intestinal tissues were fixed in a 2.5% glutaraldehyde solution for 2 h, washed with PBS, and then fixed in a 1% osmium tetroxide solution for 1.5 h. Nanosized sections were prepared after coating, stained with uranium acetate and lead citrate, and washed with distilled water. The cells were observed under a transmission electron microscope (JEM-F200; JEOL, Tokyo, Japan).

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

TRIzol (Thermo Fisher Scientific, Waltham, USA) was used to extract total RNA from the intestinal tissue according to the manufacturer's instructions. The RNA was then reverse-transcribed into complementary DNA using the RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo Fisher Scientific). RT-qPCR was performed on the QuantStudio™ 1 Plus System using the Quanti-NovaTM SYBR Green PCR Kit (208052; QIAGEN, Hilden, Germany). *U6* and *GAPDH* were used as internal controls. All RT-qPCR data were analyzed using the $2^{-\Delta\Delta CT}$ method. The primer sequences are listed in Table 1.

Western blot analysis

Intestinal tissue (50 mg) was lysed with RIPA lysis buffer, and proteins were quantified using a BCA protein quantification kit (Thermo Fisher Scientific). Protein samples were separated by SDS-PAGE and then electrotransferred onto a PVDF membrane (Millipore, Billerica, USA). The membrane was blocked with 5% skim milk for 2 h and then incubated with the primary antibody (Table 2) at 4°C overnight. The next day, the sections were incubated with the diluted secondary antibody. The images were developed using the chemiluminescence method with β-actin as the internal control. ImageJ software was used to analyze the gray values of each band, and the ratio of the gray values of the target protein to those of the internal reference protein was used to determine the expression level of the target protein.

Enzyme-linked immunosorbent assay (ELISA)

The serum indexes diamine oxidase (DAO) and intestinal fatty acid binding protein (iFABP), which reflect intestinal mucosal epithelial cell injury, were detected using the corresponding ELISA kits (Jianglai Biology, Shanghai, China), and the expression levels of serum inflammatory factors were detected using IL-1β (JL20884) and IL-18 (JL20882) kits (Jianglai, Beijing, China) according to the manufacturer's instructions.

Dual luciferase reporter assay

The biological prediction website TargetScan was used to predict the possible target genes of miR-155, and a sequence fragment containing its site of action was obtained. A luciferase reporter assay was performed using a dual luciferase reporting kit (GenaPharma, Shanghai, China). Lipofectamine 3000

Table 1. Primers used for real-time PCR

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
rno-miR-155	CGGCGGTTAATGCTAATTGTGAT	GTGCAGGGTCCGAGGT
SIRT1	ACGCCTTATCCTCTAGTTCCTGTGG	CGGTCTGTCAGCATCATCTTCCAAG
claudin-1	CTTCTGGGTTTCATCCTGGCTTCG	CCTGAGCAGTCACGATGTTGTCC
occludin	CAACGGCAAAGTGAATGGCAAGAG	TCATCCACGGACAAGGTCAGAGG
U6	CAGCACATATACTAAAATTGGAACG	ACGAATTTGCGTGTCATCC
GAPDH	GGAGTCTACTGGCGTCTTCAC	ATGAGCCCTTCCACGATGC

Table 2. Antibodies used for western blot analysis

Antibody	Manufacturer	Catalog number	Dilution
SIRT1	Wanleibio	WL02995	1:1000
claudin-1	Proteintech	28674-1-AP	1:4000
occludin	Proteintech	27260-1-AP	1:4000
NLRP3	Wanleibio	WL02635	1:1000
caspase-1	Proteintech	22915-1-AP	1:1000
ASC	Wanleibio	WL02462	1:500
GSDMD	Affinity	AF4012	1:650
NF-κB p65	Affinity	AF5006	1:1000
Acetyl-NF-kappaB p65 (Lys310)	Affinity	AF1017	1:500
β-actin	Proteintech	20536-1-AP	1:5000
Goat anti-rabbit IgG (HRP)	Proteintech	SA00001-2	1:8000

(L3000001; Invitrogen) was used to transfect HT293 cells with the SIRT1-WT and SIRT1 MUT reporter genes, and the miR-155-mimic and miR-155-inhibitor lentiviruses (Hanbio, Shanghai, China) were used to transfect the cells. The relative luciferase activity and Renilla fluorescence of the fireflies were measured 48 h after transfection.

Statistical analysis

SPSS 27.0 (SPSS, Inc., Chicago, USA) and GraphPad Prism 9.0 (GraphPad Software, La Jolla, USA) were used for data analysis. Data are presented as the mean ± standard deviation. Unmatched *t* tests were used for comparisons between two groups, and ANOVA was used for comparisons among multiple groups. $P<0.05$ was considered to indicate statistical significance. All experiments were repeated three times.

Results

Establishment of an intestinal injury model in sepsis

To demonstrate the effects of sepsis on the intestinal tract of rats, we first evaluated the relevant indicators of intestinal barrier function and the corresponding pathological changes in rats with sepsis. Compared with those in the sham group, the intestinal villi and glandular structures of the rats in the CLP group were worse, and inflammatory cell infiltration was detected via HE staining (Figure 1A), as evidenced by the elevated Chiu scores in the CLP group (Supplementary Figure S1). In addition, the levels of iFABP and DAO were higher in the serum of the CLP group than in the serum of the sham group (Figure 1B), indicating increased intestinal barrier permeability. Tight junction proteins, mainly occludin and claudin-1, help maintain intestinal barrier function. Western blot analysis, RT-qPCR, and IHC results (Figure 1C,D and Supplementary Figure S2) revealed that the protein and mRNA levels of claudin-1 and occludin were reduced in sepsis-induced intestinal tissues. These findings indicated that our CLP model was successfully established and that it induced intestinal dysfunction in experimental rats.

Sepsis induces upregulation of miR-155, decrease in SIRT1 mRNA and protein levels, and pyroptosis in rat intestinal tissues

To explore the role of miR-155 and SIRT1 in intestinal dysfunction during sepsis, the expression of miR-155 was measured via RT-qPCR, and the level of SIRT1 was measured via western blot analysis, RT-qPCR, and IHC. Compared with that in the sham group, the expression level of miR-155 in the intestinal tissue of rats in the CLP group was significantly greater, whereas the mRNA expression level of SIRT1 was decreased (Figure 1E). To further explore the protein expression level of SIRT1, western blot analysis and IHC experiments were conducted, and the results indicated that the CLP model rats exhibited significant SIRT1 downregulation (Figure 1F,G). Intestinal epithelial cell morphology, in which pyroptosis was observed, was examined using TEM. Compared with those in the sham group, the intestinal epithelial cells in the CLP group exhibited damaged cell membrane integrity, unclear mitochondrial structure, cell swelling, and nuclear shrinkage (Figure 1H). We further detected pyroptosis-related proteins, including NLRP3, pro-caspase-1, caspase-1, ASC, GSDMD and its N-terminal fragments (GSDMD-N), IL-1β, and IL-18 in intestinal tissues by western blot analysis and ELISA. The results indicated that the expressions of NLRP3, ASC, caspase-1, the GSDMD N-terminus, IL-1β, and IL-18 in the CLP group were upregulated, suggesting the occurrence of pyroptosis during sepsis (Figure 1I and Supplementary Figure S3). These results suggest that when intestinal injury occurs in sepsis, the expression of miR-155 increases, whereas SIRT1 expression decreases, accompanied by pyroptosis.

Regulation of miR-155 expression can affect intestinal barrier function and pyroptosis in septic rats

To clarify the role of miR-155 in sepsis-induced intestinal barrier dysfunction, we intraperitoneally injected rats with an AAV corresponding to miR-155 to regulate the expression level of miR-155. We assessed the extent of intestinal pathological damage, inflammatory responses, and changes in intestinal barrier function in these animals. Compared with that in the CLP group, intestinal function was improved in the CLP+AAV-miR-155-inhibitor group, as indicated by improvements in intestinal epithelial cell morphology, as shown by HE-stained sections (Figure 2A); increased levels of intestinal tight junction proteins (Figure 2B); increased percentages of areas with claudin-1 and occludin expression (Figure 2C); and reductions in the levels of iFABP and DAO in the serum (Figure 2D). However, compared with that in the CLP group, miR-155 overexpression led to increased intestinal injury. Cell pyroptosis was also decreased, which was related to the inhibition of miR-155, as indicated by decreased expressions of NLRP3, caspase-1, ASC,

GSDMD-N, IL-1β, and IL-18, as shown by western blot analysis, ELISA, and IF (Figures 1I and Figures 3A,B). Intestinal epithelial cell morphology was also observed via TEM and manifested as reduced damage to the cell membrane and mitochondria (Figure 3C). These

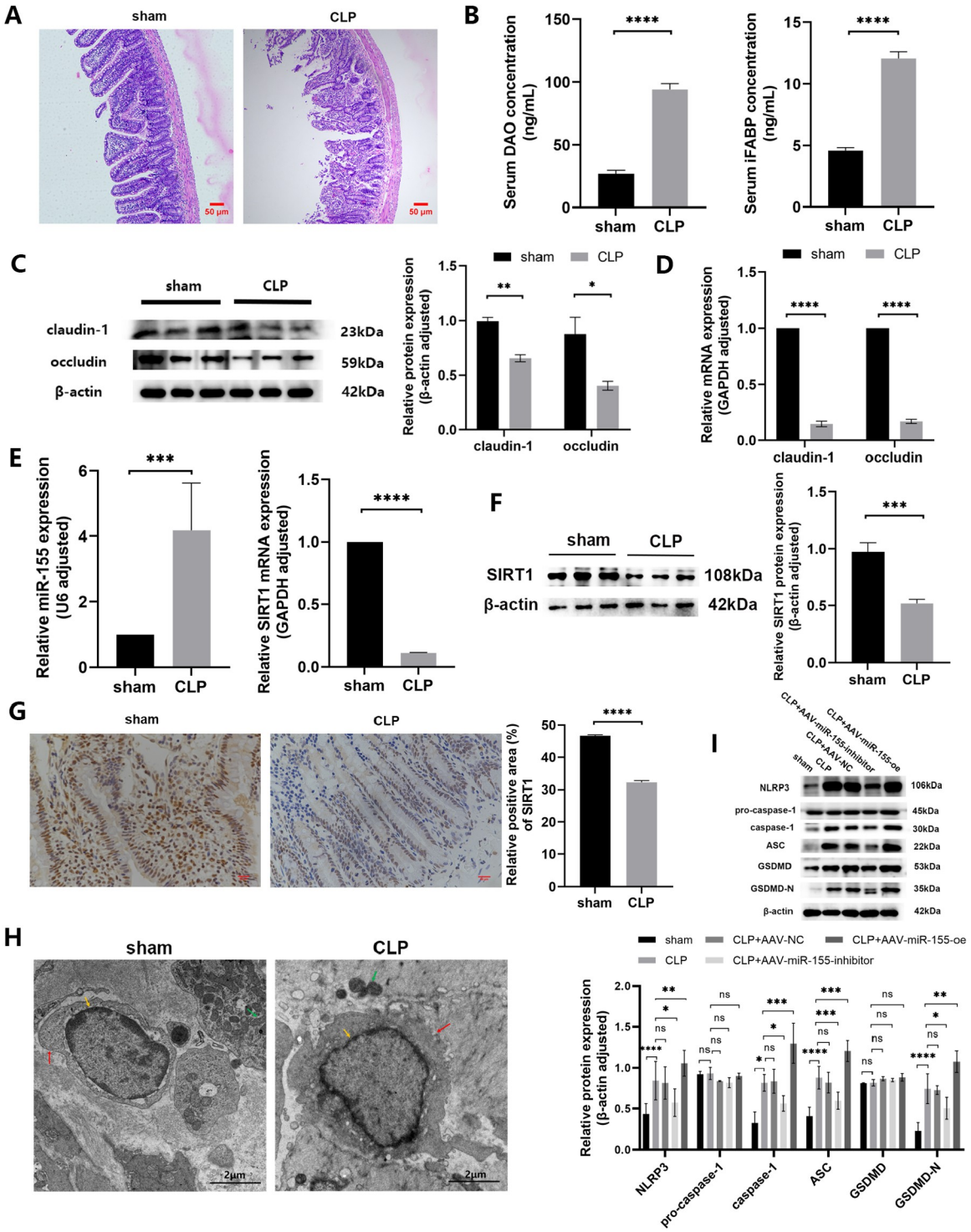

Figure 1. Sepsis induces intestinal barrier dysfunction and pyroptosis in rat intestinal tissues (A) Histopathological analysis of intestinal tissue by H&E staining (100×). (B) Serum levels of iFABP and DAO in rats were detected by ELISA. (C) The protein expressions of claudin-1 and occludin. (D) The mRNA expressions of claudin-1 and occludin. (E) The expression of miR-155 and the mRNA expression of *SIRT1*. (F) The protein expression of SIRT1. (G) Immunohistochemical staining of SIRT1 in intestinal tissues (400×). (H) The morphology of intestinal epithelial cells in the sham group and CLP group was observed by TEM (2000×). The membrane (red arrow), nucleus (yellow arrow), and mitochondria (green arrow) are shown. (I) The protein expressions of pyroptosis-related proteins. Data are presented as the mean±standard deviation (SD). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

results demonstrate that miR-155 is involved in the occurrence and development of intestinal barrier dysfunction in sepsis and that the protective effect of AAV-miR-155-inhibitor on sepsis-induced intestinal dysfunction might be achieved by inhibiting pyroptosis.

SIRT1 is a target gene of miR-155, and miR-155 inhibits the expression of SIRT1

To explore the mechanism of miR-155 in intestinal injury during sepsis, we first identified SIRT1 as a possible target of miR-155 using TargetScan software (www.Targetscan.org) (Figure 4A). A dual

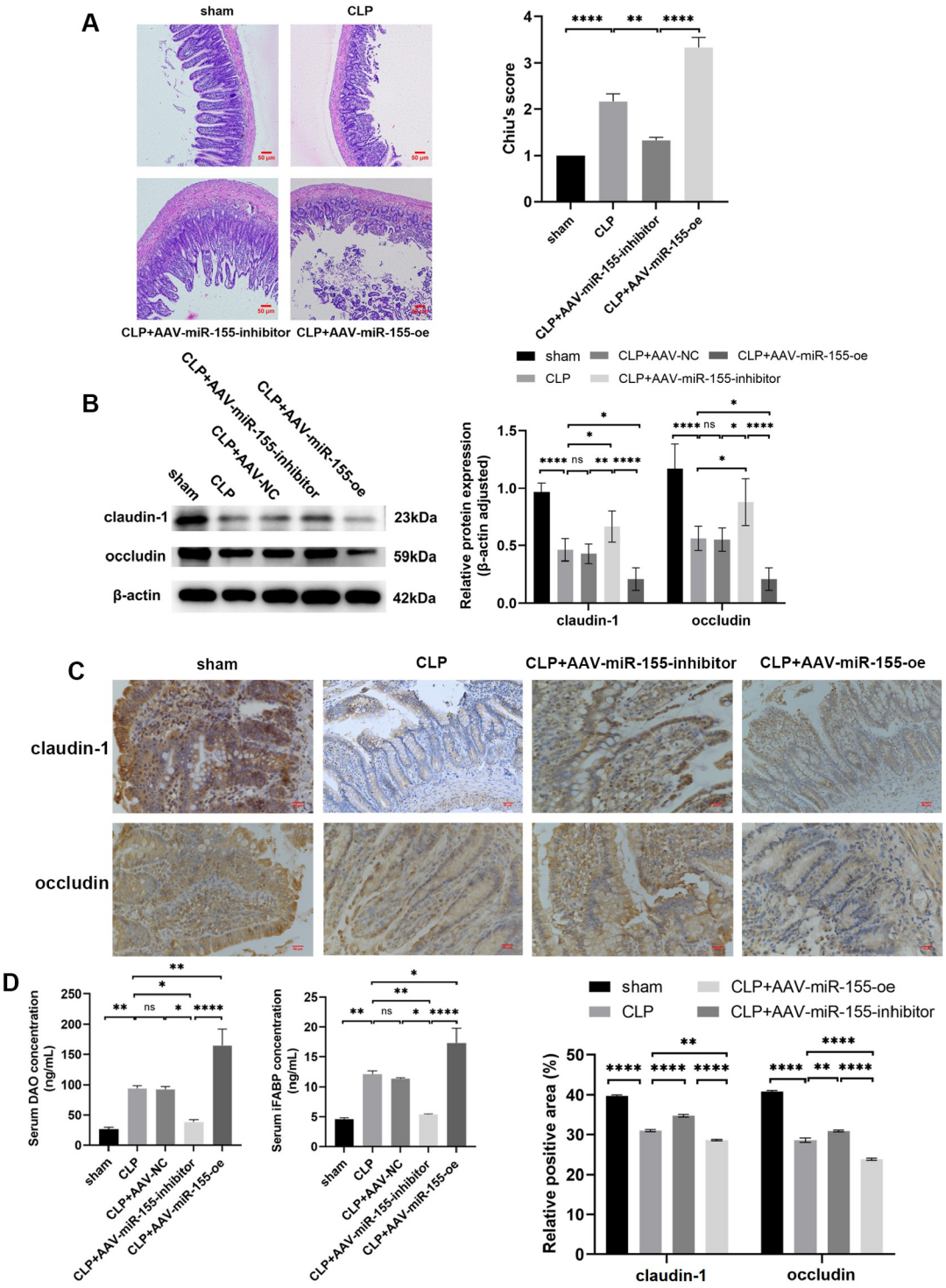

Figure 2. miR-155 promotes intestinal barrier dysfunction (A) Histopathological analysis of intestinal tissue by H&E staining (100×). (B) The protein expression and statistical analysis of claudin-1 and occludin in each group. (C) Expressions of claudin-1 and occludin in the intestinal tissue of rats in each group determined by IHC (400×). (D) Serum levels of iFABP and DAO in rats were detected by ELISA. Data are presented as the mean±standard deviation (SD). ns: not statistically significant. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

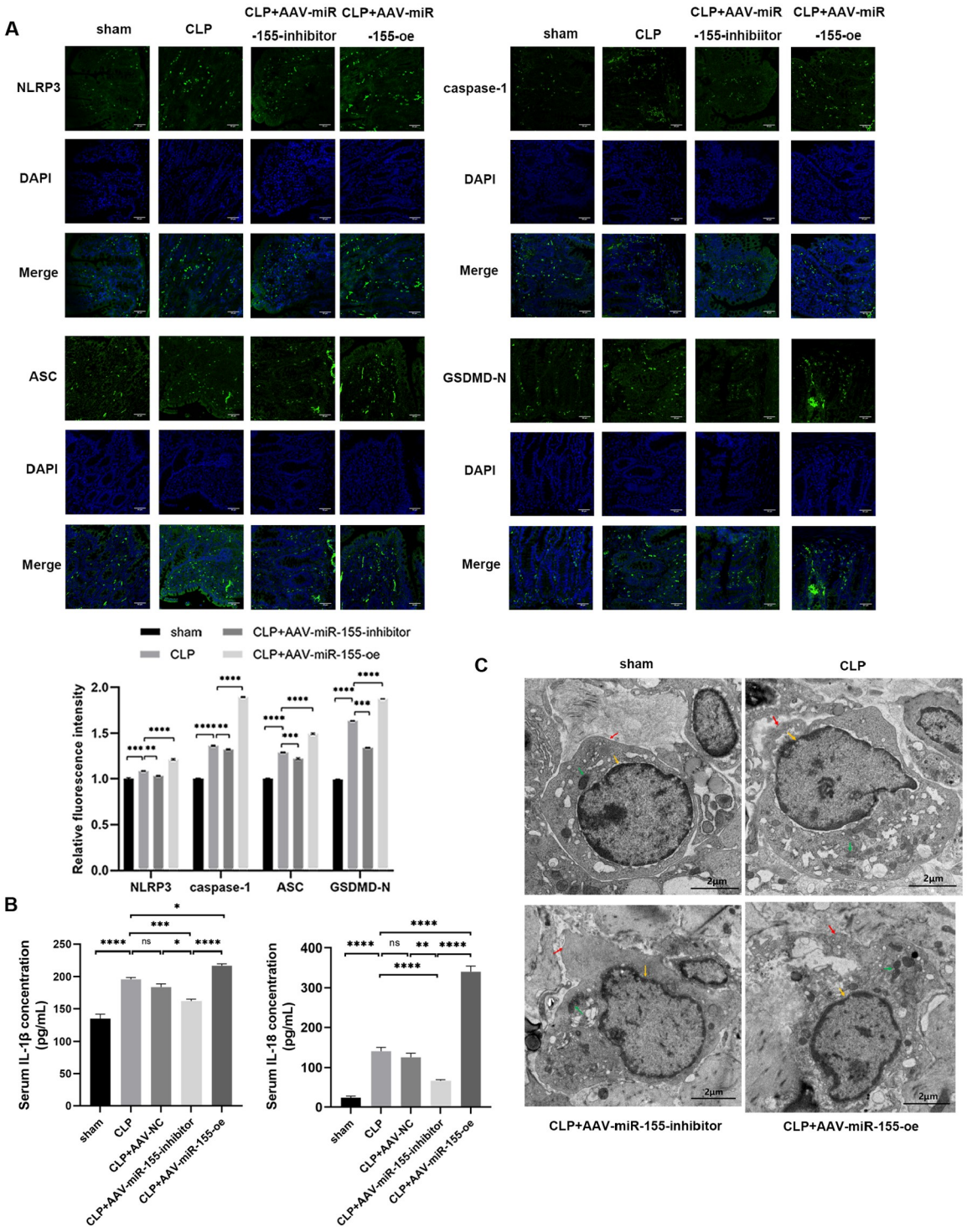

Figure 3. miR-155 promotes NLRP3 inflammasome-mediated pyroptosis in sepsis (A) Immunofluorescence staining of NLRP3, caspase-1, ASC, and GSDMD-N in intestinal tissues. (B) Serum levels of IL-1β and IL-18 in rats were detected by ELISA. (C) The morphology of intestinal epithelial cells in each group was observed by TEM (2000×). The membrane (red arrow), nucleus (yellow arrow), and mitochondria (green arrow). Data are presented as the mean±standard deviation (SD). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

luciferase reporter system further verified the binding site between miR-155 and SIRT1. The results showed that miR-155 mimics significantly reduced the fluorescence activity of SIRT1-WT but had no significant effect on the fluorescence activity of SIRT1-MUT (Figure 4B). We measured the expression levels of SIRT1 in intestinal tissues by western blot analysis, RT-qPCR, and IHC. As shown in Figure 4C,D, after the occurrence of intestinal injury in sepsis, the expression level of SIRT1 increased after the inhibition of

miR-155 expression compared with that in the CLP group, while the expression level of SIRT1 decreased after the overexpression of miR-155. The IHC results were consistent with the western blot analysis and RT-qPCR results (Figure 4E). These results suggest that SIRT1 is

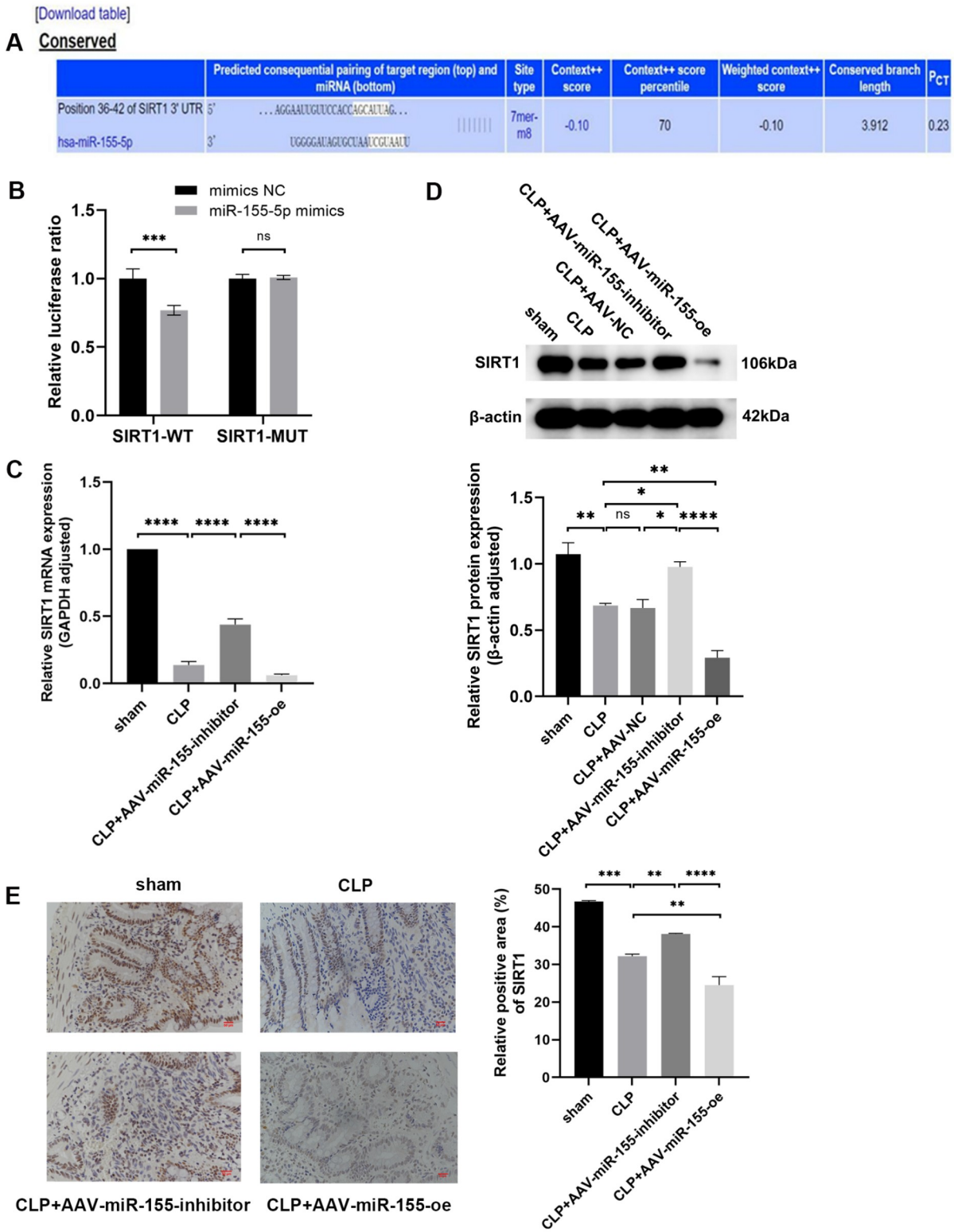

Figure 4. SIRT1 is a target of miR-155 (A) SIRT1 was confirmed to be a target of miR-155 by the TargetScan website. (B) A dual-luciferase reporter assay showed that SIRT1 is a target of miR-155. (C,D) The mRNA and protein expressions of SIRT1 in each group. (E) IHC staining was used to detect the relative area positively stained for SIRT1 (400×). Data are presented as the mean±standard deviation (SD). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

a target of miR-155, downregulating the expression level of SIRT1.

SIRT1 is a mediator of miR-155-induced intestinal dysfunction and pyroptosis in sepsis

To evaluate whether SIRT1 regulates sepsis-induced intestinal dysfunction, SRT1720 or EX-527 was intraperitoneally injected 2 h after CLP. After the administration of SRT1720, the results showed that SIRT1 overexpression markedly increased the gene expression of SIRT1. However, administration of the SIRT1 inhibitor EX-527 significantly decreased the expression level of SIRT1 (Figure 5A).

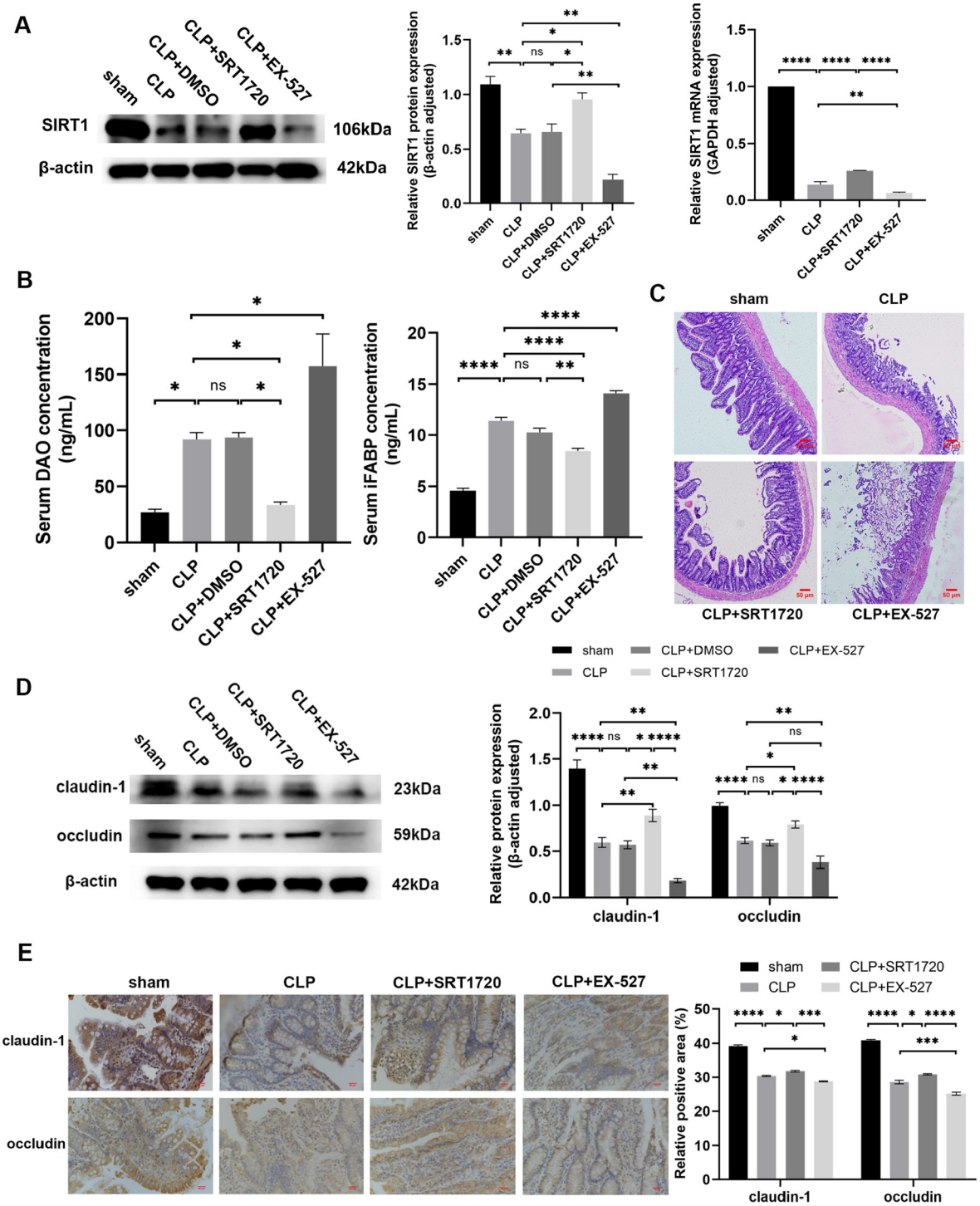

Figure 5. SIRT1 is a mediator of miR-155-induced intestinal dysfunction (A) The protein and mRNA expressions of SIRT1 in each group. (B) Serum levels of iFABP and DAO in rats were detected by ELISA. (C) Histopathological analysis of intestinal tissue by H&E staining (100×). (D) The protein expression and statistical analysis of claudin-1 and occludin in each group. (E) IHC staining was used to detect the relative positive areas of claudin-1 and occludin (400×). Data are presented as the mean±standard deviation (SD). ns: not statistically significant. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

The SRT1720 group exhibited significantly reduced intestinal villus damage, alleviated intestinal permeability increase, improved intestinal tight junctions, and reduced inflammatory factor release. However, the EX-527 group exhibited increased intestinal damage (Figure 5B–E). These results showed that the upregulation of SIRT1 could relieve intestinal damage.

To evaluate whether SIRT1 regulates sepsis-induced pyroptosis, we next conducted a series of experiments to explore the underlying mechanism involved. SRT1720 markedly inhibited the expressions of pyroptosis-related proteins, including NLRP3, caspase-1, ASC, GSDMD-N, IL-1β, and IL-18, in intestinal tissues and serum compared with those in the CLP group, while EX527 aggravated pyroptosis (Figure 6A–C, and Supplementary Figures S4 and S5), suggesting that SIRT1 plays an essential role in the initiation of pyroptosis during sepsis-induced intestinal dysfunction.

Given that NF-κB activity is responsible for the expression of proinflammatory cytokines, we examined whether SIRT1 mediates miR-155-mediated regulation of NF-κB activity in the CLP model. The inhibition of miR-155 expression was associated with increased SIRT1 expression, promoted the deacetylation of p65, and significantly downregulated p65 acetylation (Figure 6D). This is an interesting observation because the p65 subunit of NF-κB is susceptible to regulation by SIRT1, which can rapidly terminate NF-κB p65 transcription by deacetylating NF-κB p65 [24]. In contrast, reducing SIRT1 expression level with EX-527 resulted in stronger NF-κB signaling pathway activation, specifically a significant increase in the protein level of the NF-κB acetylated p65 subunit (Figure 6E and Supplementary Figure S6). These results suggest that SIRT1, a downstream target, mediates miR-155 to regulate intestinal damage during sepsis, partly by modulating p65 deacetylation and NF-κB activity.

Reduced pyroptosis caused by the miR-155 inhibitor is reversed by the SIRT1 inhibitor

To explore the key role of SIRT1 in miR-155-regulated intestinal cell pyroptosis, septic rats were treated with an AAV-miR-155-inhibitor and EX-527. The expression levels of SIRT1, inflammatory factors, and pyroptosis-related proteins were also measured. As shown in Figure 7A–C, compared with that in the CLP group, the level of SIRT1 in the AAV-miR-155-inhibitor group was significantly greater. However, when AAV-miR-155-inhibitor and EX-527 were coadministered, EX-527 decreased the AAV-miR-155-inhibitor-induced increase in SIRT1 expression. EX-527 also reversed the AAV-miR-155-inhibitor-induced decrease in acetyl-p65 (Figure 7D). Further experiments revealed that, in contrast to those in the CLP+AAV-miR-155-inhibitor group, the expression levels of pyroptosis-related proteins increased in the CLP+AAV-miR-155-inhibitor+EX-527 group, as shown by western blot analysis and IF (Figure 8A,B), and the levels of IL-1β and IL-18 were greater in the CLP+AAV-miR-155-inhibitor+EX-527 group than in the CLP+AAV-155-inhibitor group (Figure 8C). These data indicate that miR-155 inhibitor-reduced pyroptosis is reversed by the SIRT1 inhibitor.

Discussion

In this study, we established an animal model of CLP-induced intestinal damage. We evaluated the stability of our model by assessing the degree of intestinal villus damage and the levels of intestinal tight junction proteins and inflammatory factors. Our results suggest that intestinal mucosal barrier dysfunction in sepsis is accompanied by increased miR-155, decreased SIRT1, NF-κB signaling pathway activation, and pyroptosis. However, treatment with an AAV-miR-155 inhibitor or SRT1720 exerted protective effects against CLP-induced pyroptosis and intestinal barrier dysfunction. However, elevating miR-155 level or inhibiting the activation of SIRT1 had opposite effects. Moreover, we found that administration of the SIRT1 inhibitor EX-527 decreased the intestinal protection induced by the AAV-miR-155 inhibitor, which may be related to the downregulation of SIRT1 and activation of the downstream NF-κB signaling pathway. We additionally validated SIRT1 as a target of miR-155 by a dual luciferase reporter assay and confirmed that SIRT1 is a target of miR-155. Therefore, based on our findings, we propose that miR-155 activates the NF-κB signaling pathway by targeting SIRT1 and blocking p65 deacetylation, which is involved in the occurrence of intestinal pyroptosis and further aggravates intestinal mucosal barrier dysfunction in sepsis. Our results suggest that the miR-155/SIRT1/NF-κB signaling pathway may be a potential molecular target to interfere with the occurrence and development of intestinal mucosal barrier dysfunction in sepsis, thus providing a new idea for the pathogenesis and treatment of intestinal mucosal barrier dysfunction in sepsis.

Intestinal damage is a common complication of sepsis that can easily lead to bacterial displacement and multiple organ failure [27]. Pyroptosis is an inflammatory programmed cell death mechanism that differs from apoptosis and necrosis. Generally, pyroptosis can protect the host from invasive pathogens and microorganisms, but excessive pyroptosis can lead to sepsis or septic shock [28]. Pyroptosis can lead to cell death, intestinal inflammatory reactions and intestinal injury, and the inhibition of pyroptosis can reduce pathological injury [29]. The core of pyroptosis is the activation of NLRP3 and the cleavage of the GSDMD protein, which leads to the cleavage of the cell membrane and the release of intracellular substances, inducing inflammation [30]. Through *in vivo* animal experiments, we found that intestinal injury in sepsis is accompanied by pyroptosis, which is manifested by the activation of the NLRP3 inflammasome and an increase in the levels of the pyroptosis-related proteins caspase-1, ASC, and GSDMD-N. However, the underlying mechanisms require further investigation.

Several studies have shown that miRNAs regulate pathological processes related to intestinal diseases by regulating inflammatory responses and immune processes [31,32]. This study revealed that miR-155 expression was significantly elevated in the intestinal tissues of rats with septic intestinal injury. The expression of miR-155 was positively correlated with the expression of inflammatory factors. To investigate whether miR-155 plays a regulatory role in the pyroptosis of intestinal epithelial cells, we inhibited the expression of miR-155 by intraperitoneal injection of AAV-miR-155-inhibitor in animals and found that the expression of pyroptosis-related proteins decreased. Reducing the expression level of miR-155 can improve intestinal barrier dysfunction caused by sepsis and NLRP3-dependent pyroptosis. These preliminary studies and our experimental results confirm the altered expression and functional role of miR-155 in sepsis-induced intestinal mucosal barrier dysfunction; however, the underlying molecular mechanisms require further investigation. Our subsequent experiments demonstrated that miR-155 overexpression inhibited the expression of SIRT1 in sepsis-induced intestinal mucosal barrier dysfunction, whereas *miR-155* knockdown reversed this effect. Next, we identified SIRT1 as a possible target of miR-155 using the

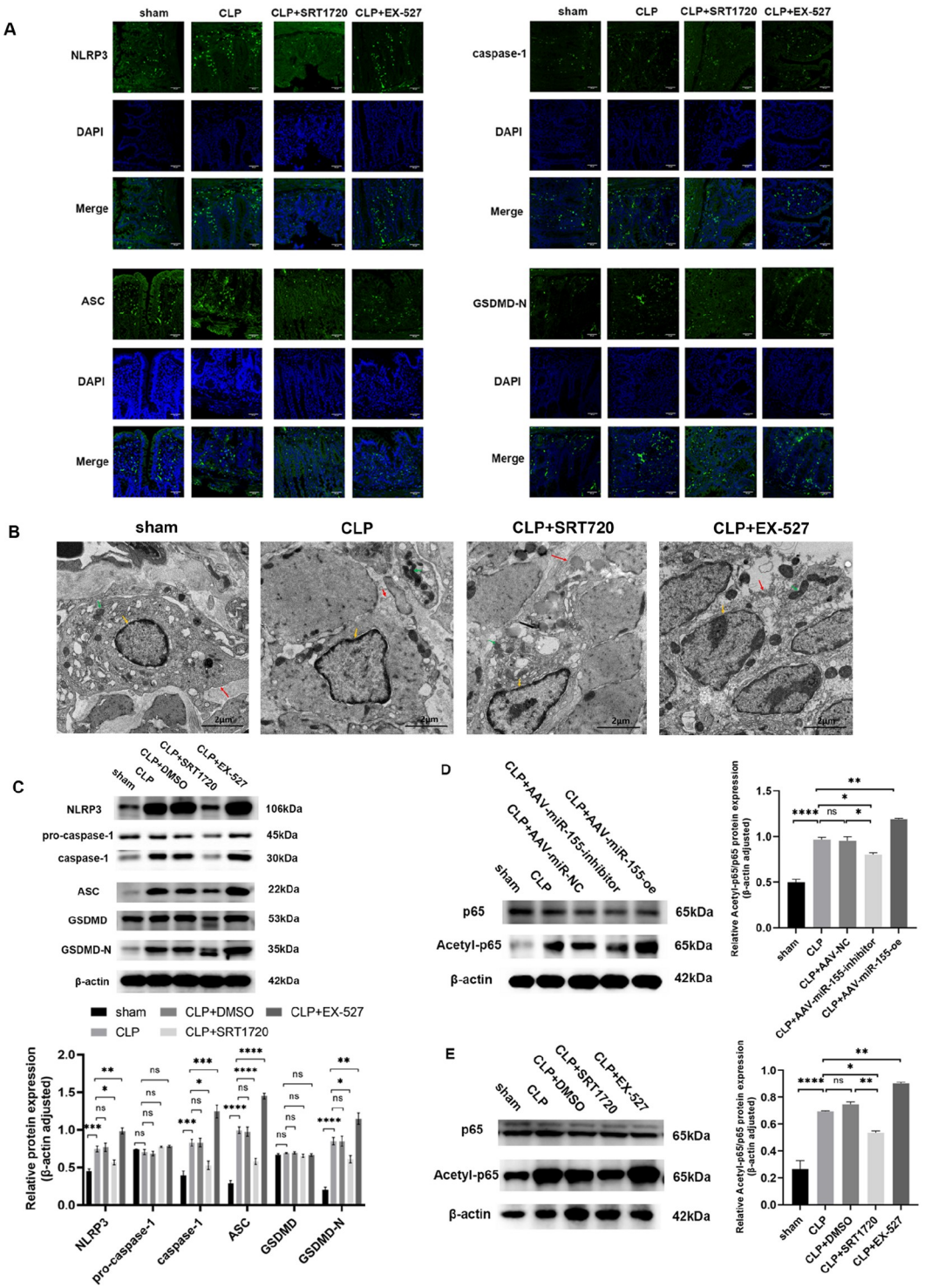

Figure 6. SIRT1 is a mediator of miR-155-induced intestinal pyroptosis in sepsis (A) Immunofluorescence staining of NLRP3, caspase-1, ASC, and GSDMD-N in intestinal tissues. (B) The morphology of intestinal epithelial cells in each group was observed by TEM (2000×). The membrane (red arrow), nucleus (yellow arrow), and mitochondria (green arrow). (C) The protein expressions of pyroptosis-related proteins in each group. (D) The protein expression of p65 and acetyl-p65 in each group. Data are presented as the mean±standard deviation (SD). ns: not statistically significant. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

TargetScan website and performed a dual luciferase reporter assay. Finally, we confirmed that SIRT1 is a target of miR-155. SIRT1, a class III histone deacetylase dependent on NAD+, can regulate immune and inflammatory responses in sepsis, control inflammatory damage in multiple organs, and ultimately protect against multiple organ dysfunction [33]. Moreover, recent studies have shown that SIRT1 can play an important role in protecting organs by regulating pathways related to pyroptosis [34]. In this study, we

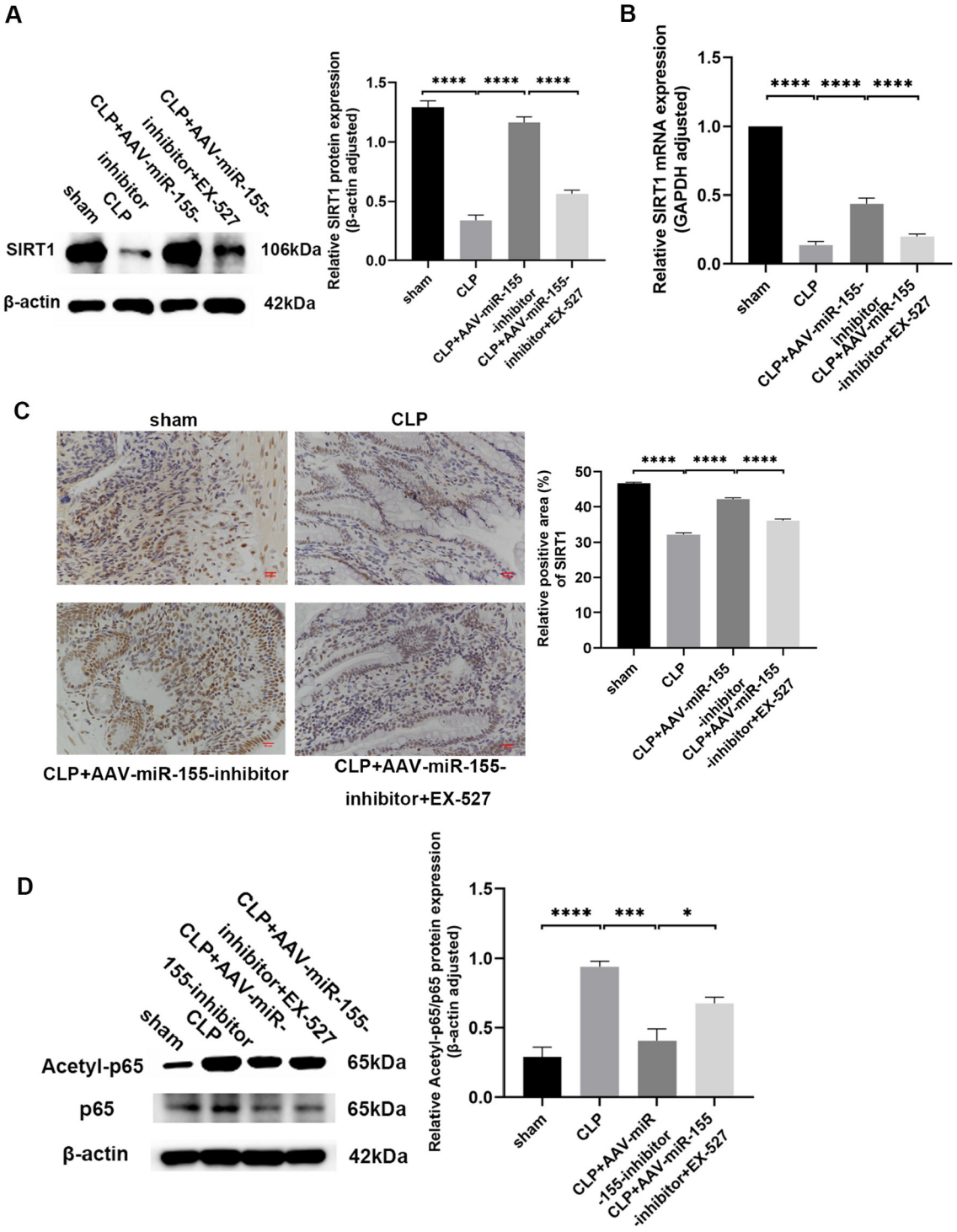

Figure 7. Downregulation of SIRT1 reverses the intestinal protective effects of miR-155 inhibitors on sepsis (A,B) The protein and mRNA expressions of SIRT1 in each group. (C) IHC staining was used to detect the relative area positively stained for SIRT1 (400×). (D) The protein expression of p65 and acetyl-p65 in each group. Data are presented as the mean±standard deviation (SD). **P*<0.05, ****P*<0.001, *****P*<0.0001.

separately administered SIRT1 inhibitors and agonists to rats with sepsis and found that SRT1720 significantly improved intestinal damage, reduced the release of inflammatory factors, and induced pyroptosis. To determine whether miR-155 regulates the occurrence of pyroptosis by targeting SIRT1, we simultaneously inhibited the expressions of miR-155 and SIRT1 and found that inhibiting the expression of SIRT1 significantly reversed the protective effect of low expression of miR-155 on the pyroptosis of intestinal epithelial cells and promoted the expressions of pyroptosis-related genes. Among the various substrates of SIRT1, NF-κB is an important

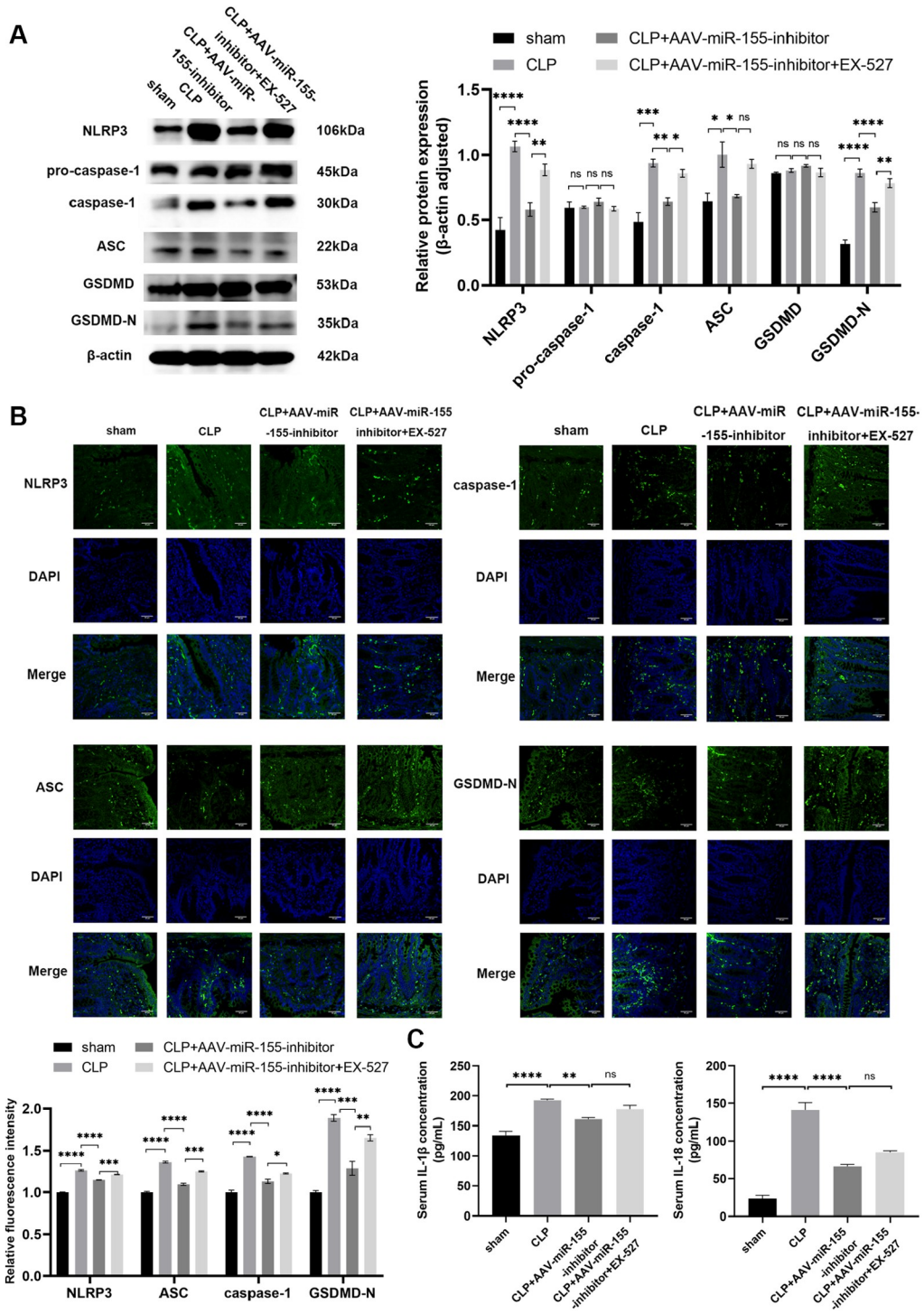

Figure 8. Downregulation of SIRT1 exacerbates intestinal pyroptosis in sepsis (A) The protein expressions of pyroptosis-related proteins in each group. (B) Immunofluorescence staining of NLRP3, caspase-1, ASC, and GSDMD-N in intestinal tissues. (C) Serum levels of IL-1β and IL-18 in rats were detected by ELISA. Data are presented as the mean±standard deviation (SD). ns: not statistically significant. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

transcription factor involved in eukaryotic inflammatory responses, cell proliferation, and apoptosis [23]. SIRT1 inhibits the NF-κB signaling pathway through the deacetylation of the NF-κB subunit p65 at lysine 310, thereby reducing the production of inflammatory factors and chemokines [24]. To determine whether SIRT1 directly regulates the acetylation level of p65 and affects the occurrence of pyroptosis in intestinal epithelial cells, we performed western blot analysis experiments and found that overexpression of SIRT1 reduced the acetylation level of p65, whereas the inhibition of SIRT1 promoted the acetylation of p65. To verify how SIRT1 regulates the

transcriptional activity of p65, we conducted IHC experiments and found that the expression of p65 in the nucleus significantly decreased after SIRT1 overexpression, suggesting that SIRT1 partially inhibits the nuclear translocation of p65 by deacetylating p65, thereby inhibiting its transcriptional activity. In sepsis, the expressions of NLRP3, IL-1β, and IL-18 are decreased, the pyroptosis of intestinal epithelial cells is inhibited, and the intestinal mucosal barrier dysfunction is subsequently improved.

In this study, we hypothesized that miR-155 induces intestinal barrier damage by promoting pyroptosis via the SIRT1/NF-κB signaling pathway in sepsis. Our experiments also revealed that SRT1720 and EX-527 may regulate pyroptosis during sepsis-related intestinal damage. SIRT1 downregulation exacerbates intestinal damage and pyroptosis in sepsis and reverses the intestinal protective effects of miR-155 inhibitors in septic rats.

In summary, our results demonstrate that miR-155 can reduce the deacetylation of the NF-κB subunit p65 by targeting SIRT1, thereby increasing NF-κB signaling activity and enhancing the occurrence of NLRP3 inflammasome-mediated pyroptosis, leading to intestinal mucosal barrier dysfunction in sepsis. These results provide new insights into the role of miR-155 in regulating the pathogenesis of intestinal mucosal barrier dysfunction in sepsis and may contribute to the treatment of intestinal mucosal barrier dysfunction in sepsis. However, the effectiveness of these biomarkers and targets for diagnosing and predicting sepsis remains to be demonstrated.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biphysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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