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**Gut Microbiota-Derived Hexanoic Acid Mediates the Protective Effects of Electroacupuncture
Against Sepsis Through Macrophage Reprogramming**

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Highlights:

1. Electroacupuncture alleviates sepsis-associated liver injury by promoting the gut microbial *Lactobacillus vaginalis*–hexanoic acid axis.
2. Microbiota-derived hexanoic acid targets BMAL1 to suppress NF-κB-mediated pro-inflammatory macrophage polarization.
3. INT-777-functionalized pH-responsive liposomes enhance the hepatic delivery and therapeutic efficacy of hexanoic acid.

Abstract

Sepsis-induced liver injury (SLI) is a severe complication of sepsis with limited effective therapeutic options. This study explored the mechanism by which electroacupuncture (EA) protects against SLI and its therapeutic translation through a targeted nanodelivery system. Our findings showed that EA remodeled the gut microbiota, increased the abundance of *Lactobacillus vaginalis* (*L.V*), and promoted the production of microbiota-derived hexanoic acid (HA). Mechanistically, HA interacted with BMAL1 and inhibited NF- κ B activation, thereby suppressing pro-inflammatory macrophage polarization and alleviating hepatic inflammation and injury. However, the rapid metabolism and limited therapeutic delivery of free HA may restrict its therapeutic application. To overcome these limitations, we developed an INT-777-modified PH-responsive liposomal nanodelivery system (HA@L-INT) to enhance the targeted delivery of HA. HA@L-INT exhibited enhanced hepatic accumulation and superior therapeutic efficacy compared with free HA, resulting in greater suppression of inflammatory macrophage polarization and liver injury. Collectively, the gut microbial *L.V*-HA axis mediates the protective effects of EA against SLI by regulating macrophage polarization, while targeted nanodelivery further enhances the therapeutic potential of HA.

[Key Words] Electroacupuncture, Sepsis-induced liver injury, *Lactobacillus. Vaginalis*, Hexanoic acid, BMAL1.

Abbreviations: Alcian blue-periodic acid-Schiff staining: AB-PAS; Alanine aminotransferase: ALT; Aspartate aminotransferase: AST; Brain and muscle ARNT-like protein 1: BMAL1; Circadian locomotor output cycles kaput: CLOCK; Electroacupuncture: EA; Fecal microbiota transplantation: FMT; Hematoxylin and eosin: H&E; Interferon-gamma: IFN- γ ; Interleukin-1 beta: IL-1 β ; Interleukin-6: IL-6; Interleukin-10: IL-10; INT-777-modified pH-responsive liposomes: @L-INT; *Lactobacillus vaginalis*: *L.V*; Lipopolysaccharide: LPS; Sepsis-induced liver injury: SLI; Surface plasmon resonance: SPR; Tumor necrosis factor-alpha: TNF- α ; Western blot: WB; Zonula occludens-1: ZO-1; Zusanli: ST36.

Introduction

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection and commonly occurs following severe trauma, burns, shock, or major surgery, representing a major global public health concern(Cao et al., 2023; Singer et al., 2026). SLI is one of the most common and severe forms of organ damage during sepsis progression and has been recognized as an independent predictor of poor prognosis in patients with sepsis(Guo et al., 2025; Xu et al., 2025). The gut–liver axis is a bidirectional regulatory network involving the gut microbiota, intestinal mucosal barrier, and immune cells(Pabst et al., 2023). During sepsis, intestinal hypoperfusion and epithelial barrier disruption, together with gut microbial dysbiosis, facilitate the overgrowth of opportunistic pathogens and the translocation of bacteria and endotoxins into the portal circulation(Hsu and Schnabl, 2023; Tian and Li, 2025). These gut-derived microbial products activate hepatic Kupffer cells and downstream pro-inflammatory signaling pathways, thereby amplifying hepatic inflammation and aggravating hepatocellular injury (Tilg et al., 2022; Zheng et al., 2026). Therefore, restoring gut–liver axis homeostasis represents a promising therapeutic strategy for SLI.

EA is a non-pharmacological intervention that combines traditional acupuncture with controlled electrical pulse stimulation(Xiong et al., 2026). In recent years, the protective effects of EA against sepsis and sepsis-associated organ injury have attracted increasing attention. Studies have shown that low-intensity EA at ST36 activates somatosensory–autonomic reflexes and the vagal–adrenal axis, thereby suppressing NF- κ B signaling and pro-inflammatory cytokine production (Liu et al., 2021). Lu et al. employed high-spatiotemporal-resolution intravital three-dimensional imaging to reveal that EA at ST36 effectively restrained pathological neutrophil aggregation and swarming in lipopolysaccharide-induced septic mice, providing direct visual evidence of the anti-inflammatory effects of EA in vivo(Lu et al., 2026). The present study further reveals a gut microbiota–metabolite–circadian clock regulatory mechanism underlying the protective effects of EA against SLI. Specifically, EA increases gut microbiota-derived acetate, promotes BMAL1 expression, and suppresses NF- κ B signaling, thereby modulating M1 macrophage polarization.

However, the therapeutic application of free HA is limited by its rapid metabolism and clearance in vivo, which may result in insufficient systemic exposure and reduced accumulation at inflammatory sites(Ishiwata et al., 1995; Shegani et al., 2022). Moreover, the lack of targeted delivery may further restrict its therapeutic efficacy while increasing unnecessary exposure to non-target tissues. Nanotechnology-based delivery systems provide an opportunity to overcome these limitations by improving drug stability, controlling drug release, and enhancing accumulation at pathological sites(Lee and Thompson, 2017; Tsirogianni et al., 2025). Accordingly, we developed an INT-777-modified pH-responsive liposomal nanodelivery system (HA@L-INT). Given that TGR5 is functionally expressed in macrophages and its activation by INT-777 suppresses macrophage inflammatory responses, INT-777 functionalization was incorporated to enhance the macrophage-oriented therapeutic properties of the nanoplatform (Pols et al., 2011; Wang et al., 2020). Through this strategy, we sought to translate an EA-induced microbiota-derived metabolite into a targeted nanotherapeutic approach for sepsis.

Materials and methods

Materials

Sodium hexanoate, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), N-hydroxysuccinimide (NHS), cholesterol, dioleoylphosphatidylethanolamine (DOPE), and cholesteryl hemisuccinate were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). The cationic lipid DOTAP and amino-functionalized lipid DSPE-PEG₂₀₀₀-NH₂ were obtained from Avanti Polar Lipids (Alabaster, AL, USA). INT-777, a selective TGR5 agonist, was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Dialysis membranes with a molecular-weight cutoff of 3.5 kDa were purchased from Thermo Fisher Scientific (Waltham, MA, USA), and the near-infrared fluorescent dye DiR was obtained from Beyotime Biotechnology (Shanghai, China). Anti-BMAL1 (Cat. No.14020), anti-CLOCK (Cat. No.5157), anti-NF- κ B p65 (Cat. No.8242), and anti-phospho-NF- κ B p65 (Cat. No. 3033) rabbit monoclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-Bax (Cat. No. R380709), anti-Bcl-2 (Cat. No. 250198), and anti-cleaved caspase-3 (Cat. No.341034) antibodies were obtained from Zen BioScience (Chengdu, China). anti-mouse CD206 (Cat. No.17-2061-82), anti-mouse CD86 (Cat. No. 12-0862-82), and FITC-conjugated anti-mouse F4/80 (Cat. No.11-4801-82) antibodies were purchased from Thermo Fisher Scientific. BMAL1 protein (Cat No: HY-P704046) was purchased from MCE.

Animal Model Establishment and Experimental Interventions

Male C57BL/6 mice (6-8 weeks) were obtained from Beijing Viton Lihua Laboratory Animal Technology Co., Ltd., and housed in a specific pathogen-free environment. Sepsis was induced by lipopolysaccharide (LPS) administration. Briefly, mice received a single intraperitoneal injection of LPS at a dose of 20 mg/kg, whereas mice in the control group received an equal volume of normal saline. For EA treatment, bilateral ST36, located approximately 4 mm distal to the knee joint and 2 mm lateral to the anterior tibial tubercle, were selected. Sterile acupuncture needles were inserted into bilateral ST36 to a depth of approximately 2–3 mm and connected to an electroacupuncture stimulator. Electrical stimulation was delivered using alternating frequencies of 2 and 15 Hz at an intensity of 1 mA for 30 min per session. In the sham EA group, needles were inserted superficially into bilateral non-acupoints adjacent to ST36 without electrical stimulation. For sodium hexanoate intervention, mice were administered sodium hexanoate at a dose of 500 mg/kg by oral gavage once daily for three consecutive days before LPS challenge. Mice in the corresponding vehicle control group received an equal volume of normal saline according to the same administration schedule. All animal procedures were conducted in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Ethics Committee of Hunan Provincial People's Hospital (Approval No. 2024-707).

Patients With Sepsis and Blood Sample Collection

Patients who met the Sepsis-3 diagnostic criteria and were clinically diagnosed with sepsis were enrolled in this study. Between June 2021 and June 2023, blood samples were collected from 12 patients with sepsis admitted to the intensive care unit of the Third Xiangya Hospital of Central South University. During the same period, 12 healthy volunteers were recruited as controls. All blood samples were

separated and processed within 1 h of collection and subsequently used for the measurement of inflammatory cytokines and targeted short-chain fatty acid metabolomic analysis. The study protocol was reviewed and approved by the Medical Ethics Committee of the Third Xiangya Hospital of Central South University (Approval No. 2018-S17). Written informed consent was obtained from all participants before sample collection.

Cell Culture and Model Establishment

RAW264.7 murine macrophages (ATCC, TIB-71) and AML12 murine hepatocytes (ATCC, CRL-2254) were cultured at 37 °C in a humidified atmosphere containing 5% CO₂. RAW264.7 cells were maintained in high-glucose DMEM, whereas AML12 cells were maintained in DMEM/F-12; both media were supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. To establish an inflammatory model, RAW264.7 cells were stimulated with LPS (100 ng/mL) for 24 h. Where indicated, cells were pretreated with STL1267 for 1 h and subsequently exposed to LPS in the continued presence of STL1267 for 24 h.

Bacterial Culture

Lactobacillus. vaginalis (BNCC186140) was inoculated into de Man, Rogosa and Sharpe (MRS) broth and cultured under anaerobic conditions at 37°C for 24 h. After incubation, the bacterial suspension was serially diluted, and an appropriate volume of each dilution was evenly spread onto MRS agar plates containing 1.5% agar. The plates were subsequently incubated under anaerobic conditions at 37°C for 24–48 h. Bacterial colonies were counted, and the bacterial concentration was calculated according to the corresponding dilution factor and expressed as colony-forming units per milliliter (CFU/mL).

Metabolomics, transcriptomics and 16S rRNA

Targeted short-chain fatty acid analysis was performed using a Q-Exactive Orbitrap mass spectrometer. Data preprocessing, including noise reduction, peak detection, peak extraction, and peak alignment, was conducted using MetaboScape 5.0 software. Both 16S rRNA and transcriptomic sequencing were performed on the Illumina NovaSeq platform, followed by standard downstream bioinformatic analyses.

Western blotting

Total proteins were extracted from liver tissues and cultured cells using RIPA lysis buffer supplemented with protease and phosphatase inhibitors, and protein concentrations were determined using a BCA assay. Equal amounts of protein from each group were separated by 10% SDS–PAGE and transferred onto PVDF membranes. After blocking, the membranes were incubated overnight at 4 °C with primary antibodies against p65, phospho-p65 (p-p65), BMAL1, CLOCK, Bax, caspase-3, Bcl-2, and GAPDH (1:1,000). After washing, the membranes were incubated with HRP-conjugated secondary antibodies (1:5,000) for 1 h at room temperature, and protein bands were subsequently visualized using an ECL detection reagent.

Histopathological, Immunohistochemical, and Immunofluorescence Analyses of Liver Tissues

Liver tissues were fixed in 4% paraformaldehyde for 36 h, paraffin-embedded, and sectioned. H&E staining was performed to assess hepatic architecture, hepatocyte degeneration and necrosis, and inflammatory cell infiltration. CD68 expression was evaluated by immunohistochemistry. For

immunofluorescence staining, sections were incubated with antibodies against F4/80, CD86, and CD206, followed by appropriate fluorescent secondary antibodies and DAPI counterstaining. Hepatocyte apoptosis was assessed using TUNEL staining according to the manufacturer's instructions.

Flow cytometric analysis

After the indicated treatments, RAW264.7 macrophages were collected, washed twice with pre-cooled PBS, and resuspended as a single-cell suspension. The cells were stained with fluorochrome-conjugated antibodies against F4/80, CD86, and CD206 at 4 °C for 30 min in the dark. After washing, the cells were analyzed by flow cytometry, and the data were processed using FlowJo software. F4/80⁺CD86⁺ and F4/80⁺CD206⁺ cells were defined as M1- and M2-like macrophages, respectively.

qRT-PCR

Total RNA was isolated from liver tissues and RAW264.7 macrophages using a commercial extraction kit. RNA quantity and quality were evaluated before reverse transcription, and equal amounts of RNA were converted into cDNA using the PrimeScript™ RT Reagent Kit. Real-time PCR amplification was conducted with TB Green® Premix Ex Taq™ using a StepOne™ Real-Time PCR System. Target gene expression was normalized to GAPDH and expressed as fold changes relative to the indicated control group using the 2^{-ΔΔCt} approach. Primer information is provided in Table S1.

Fecal microbiota transplantation

FMT was performed according to a previously published protocol from our group, with minor modifications (Yan et al., 2026). Briefly, antibiotic-pretreated recipient mice received fecal suspensions prepared from septic or EA-treated donor mice by oral gavage for 5 consecutive days before sepsis induction.

Molecular docking, molecular dynamics simulation, and SPR analysis

The three-dimensional structures of BMAL1 and HA were obtained from the RCSB Protein Data Bank and PubChem, respectively. Molecular docking was performed using AutoDock Vina, and the pose with the lowest predicted binding energy was visualized using PyMOL 2.5. The selected BMAL1–HA complex was subjected to a 100-ns all-atom molecular dynamics simulation using GROMACS 2022.3. Surface plasmon resonance was performed to determine the binding affinity between HA and BMAL1 by immobilizing recombinant BMAL1 protein on a CM5 sensor chip via amine coupling, using a blank flow cell as the reference, followed by the injection of serially diluted HA at 25 °C. Following reference subtraction and solvent correction, the sensorgrams were fitted to a 1:1 Langmuir model to determine the equilibrium dissociation constant (K_D).

Preparation of INT-777-modified pH-responsive cationic HA sodium lipid nanoparticles (HA@L-INT)

HA@L-INT was prepared using thin-film hydration followed by surface conjugation. Briefly, DOTAP, CHEMS, DOPE, cholesterol, and DSPE-PEG₂₀₀₀-NH₂ were dissolved in chloroform/methanol (2:1, v/v) at a molar ratio of 35:25:20:15:5. The organic solvents were removed by rotary evaporation, and the resulting lipid film was vacuum-dried overnight. The film was then hydrated with sodium hexanoate-saturated PBS (pH 7.4) at 60 °C for 1 h, sonicated, and extruded 10 times through a 200-nm polycarbonate membrane. Free sodium hexanoate was removed by dialysis against PBS (MWCO, 3.5

kDa) at 4 °C for 24 h to obtain HA@L-NH₂. For surface functionalization, INT-777 was activated with EDC and NHS at a molar ratio of 1:2:2 in DMSO for 2 h at room temperature and then added dropwise to the HA@L-NH₂ suspension. After stirring for 8 h at room temperature, the resulting HA@L-INT was purified by dialysis against PBS (MWCO, 3.5 kDa) for 24 h and stored at 4 °C until use.

Characterization

The microstructural morphology of liposomes was observed using a transmission electron microscope. The hydrodynamic diameter, polydispersity index (PDI), and surface zeta potential of the liposomes were determined using a dynamic light scattering instrument (DLS, Malvern Zetasizer Nano ZS). To validate the pH-responsive release profile, HA@L-INT was placed in a dialysis bag (MWCO: 3.5 kDa) and immersed in release media at different pH conditions (pH 7.4, simulating blood circulation; pH 5.5, simulating lysosomal and inflammatory microenvironments), followed by incubation under constant temperature oscillation conditions at 37 °C and 100 rpm. Samples were collected at predetermined time points, and their particle size and zeta potential were measured using DLS to evaluate their stability across different pH conditions.

Determination of the encapsulation efficiency (EE%) and drug loading (DL%) by ultraviolet spectrophotometry

The content of sodium hexanoate in liposomes was determined by demulsification method. An appropriate amount of purified HA@L-INT was taken, and an ultrasonic demulsification process was performed using a methanol solution containing 0.1% Triton X-100 to completely release the encapsulated sodium hexanoate. The absorbance was measured at specific characteristic absorption wavelengths using a UV-Vis spectrophotometer. A standard curve was first constructed using gradient concentrations of free sodium hexanoate standards. Based on the standard curve, the concentration of sodium hexanoate in the liposomes was calculated, and the encapsulation efficiency (EE) and drug loading (DL) were calculated according to the following formulas. $EE (\%) = (W_{\text{encapsulated}} / W_{\text{total added}}) \times 100\%$; $DL (\%) = (W_{\text{encapsulated}} / W_{\text{total lipid}}) \times 100\%$.

Cell uptake assay

FITC-labeled HA@L-INT (FITC-HA@L-INT) was prepared by incorporating FITC during liposome hydration, followed by dialysis to remove free dye. RAW264.7 cells were seeded in confocal dishes at 1×10^5 cells/dish and cultured for 24 h. The cells were then incubated with FITC-HA@L-INT at 37 °C for 4 h, washed three times with ice-cold PBS, and fixed with 4% paraformaldehyde for 15 min. Cellular lipids and nuclei were stained with Nile Red (1 µg/mL, 10 min) and DAPI (1 µg/mL, 5 min), respectively. Fluorescence images were acquired using a Leica TCS SP8 confocal laser scanning microscope.

Cytotoxicity test

RAW264.7 cells were seeded in confocal dishes or 96-well plates at a density of 1×10^4 cells/well and allowed to adhere overnight. The cells were subsequently treated with free HA (1 mM), HA@L (100 µg/mL), or HA@L-INT (100 µg/mL) for 24 h. After washing three times with PBS, the cells were stained with 2 µM Calcein-AM and 4.5 µM propidium iodide (PI) at 37 °C in the dark for 30 min. Following two additional washes with PBS, fluorescence images were immediately acquired using an inverted

fluorescence microscope. Viable and dead cells were identified by green Calcein-AM fluorescence and red PI fluorescence, respectively.

Hemolysis test

Fresh anticoagulated blood was collected from C57BL/6 mice and centrifuged at 1,000 rpm for 5 min. The isolated RBCs were washed with PBS until the supernatant was clear and diluted to 2% (v/v). Equal volumes of the RBC suspension and HA@L-INT at different concentrations were incubated at 37 °C for 1 h, with PBS and deionized water serving as negative and positive controls, respectively. After centrifugation, the absorbance of the supernatant was measured at 540 nm.

Liver-targeted distribution within the body

To track the distribution of liposomes in vivo, the fluorescent probe DiR was incorporated during the preparation process. Following LPS-induced modeling, mice were administered DiR-labeled HA@L-INT or non-targeted HA@L via tail vein injection. At 1, 3, 6, 9, and 12 hours post-injection, the mice were euthanized, and their major organs (heart, liver, spleen, lungs, kidneys) were dissected; fluorescence distribution was then observed using an in vivo imaging system for small animals (IVIS Spectrum).

Statistical analysis

All data are expressed as mean $\pm$ SD. Statistical analyses were performed using GraphPad Prism 9.0 software. Comparisons between two groups were conducted using the two-sided unpaired Student's t-test; comparisons among multiple groups were performed using one-way ANOVA followed by Tukey's post-hoc test; survival curve analysis was carried out using the Kaplan–Meier method, with differences in survival rates assessed using the Log-rank test. A *P* value <0.05 was considered statistically significant.

Result

EA Alleviates SLI

To evaluate the protective effects of EA against sepsis, an LPS-induced mouse model was established, and the mice received EA or sham EA stimulation for 1 week before model induction (Fig.1A). EA significantly reduced mortality and improved clinical scores in LPS-treated mice (Fig.1B, C). Given that the early inflammatory storm is a major contributor to sepsis-associated liver injury, serum samples were collected 24 h after LPS administration to assess the effects of EA on cytokine production. EA markedly decreased the circulating levels of pro-inflammatory cytokines, including IL-6, IL-1 β , TNF- α , and IFN- γ , while increasing the level of the anti-inflammatory cytokine IL-10 (Fig.1D). Consistent with these findings, EA significantly reduced the hepatic mRNA expression of inflammatory cytokines, serum transaminase levels, and renal function-related biochemical indices compared with the LPS group (Fig.1E-G). Gross examination showed that EA markedly improved LPS-induced changes in liver appearance, including dark discoloration and tissue congestion (Fig.1H). Histological analysis further confirmed that EA alleviated LPS-induced liver injury, as evidenced by reduced hepatic hemorrhage, edema, and macrophage infiltration (Fig. 1I). TUNEL staining showed that EA decreased hepatocyte apoptosis (Fig.1J). Moreover, transmission electron microscopy revealed that LPS induced marked hepatocellular ultrastructural damage, characterized by mitochondrial swelling, disruption of mitochondrial cristae, and extensive cytoplasmic vacuolization, whereas EA treatment preserved mitochondrial morphology and substantially alleviated these ultrastructural abnormalities (Fig. 1K).

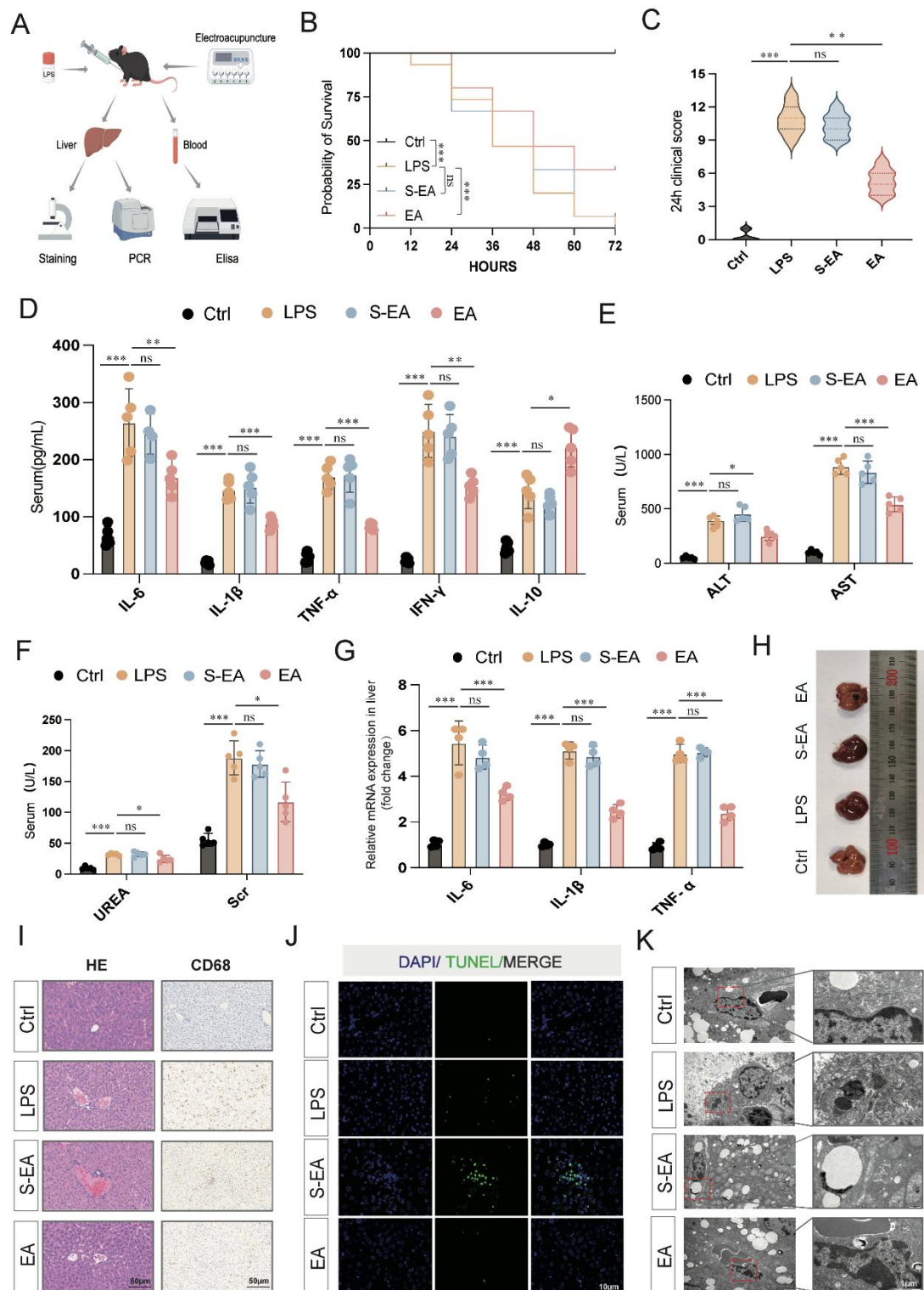


Figure 1. EA alleviates SLI. (A) Experimental protocol; (B) Kaplan–Meier survival curves; (C) Clinical scores; (D) Serum inflammatory cytokine levels; (E) Serum liver function; (F) Serum kidney function; (G) Hepatic mRNA expression of inflammatory cytokines; (H) Quantitative analysis of liver injury; (I) H&E staining and CD68 immunohistochemistry of liver tissue; (J) TUNEL staining of liver tissues; (K) TEM images of liver tissue. Data are expressed as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Transcriptome analysis revealed the mechanism of EA in the treatment of SLI

To further elucidate the molecular mechanisms underlying the protective effects of EA against SLI, we performed transcriptomic sequencing. Volcano plot analysis revealed extensive transcriptional alterations among the three groups. A total of 4,042 differentially expressed genes (DEGs) were identified between the Ctrl and LPS groups, whereas 2,718 DEGs were detected between the LPS and EA groups (Fig. 2A, B). Venn diagram analysis further identified 1,175 overlapping DEGs among the three experimental groups (Fig. 2C). Gene Ontology enrichment analysis showed that these DEGs were predominantly involved in immune system processes, responses to stimuli, and molecular transducer activity (Fig. 2D). KEGG pathway enrichment analysis indicated that the therapeutic effects of EA were closely associated with the NF- κ B signaling pathway and circadian rhythm-related pathways (Fig. 2E). To investigate the expression of circadian rhythm-related genes in patients with sepsis, we analyzed differential gene expression in the GSE57065 dataset obtained from the Gene Expression Omnibus database. The results showed that CLOCK, a core circadian regulator, was expressed at a significantly higher level in healthy controls than in patients with sepsis (Fig. 2F, G). KEGG enrichment analysis of the DEGs further identified the NF- κ B signaling pathway as a key pathway associated with sepsis (Fig. 2H). Immune-cell infiltration analysis using the Immune Cell AI algorithm revealed a marked increase in macrophage infiltration in the livers of septic mice (Fig. 2I). Consistent with these findings, single-cell RNA sequencing analysis showed that CLOCK was highly expressed in macrophages from healthy individuals, and its expression was negatively correlated with macrophage infiltration (Fig. 2J, K). Given the critical role of macrophage polarization in sepsis-induced liver injury, we subsequently assessed the proportions of M1-and M2-polarized macrophages in the liver. Immunofluorescence staining showed that pro-inflammatory M1 macrophages (CD68⁺/CD86⁺) were markedly increased in the LPS group. In contrast, EA treatment reduced M1 macrophage accumulation and promoted polarization toward the anti-inflammatory M2 phenotype (CD68⁺/CD206⁺) (Fig. 2L). Immunofluorescence staining further demonstrated that EA inhibited NF- κ B signaling activation in hepatic macrophages (Fig. 2M). Consistently, western blotting showed that EA suppressed NF- κ B pathway activation and restored the expression of the core circadian proteins BMAL1 and CLOCK compared with the LPS group (Fig. 2N).

To further determine whether SLI is influenced by circadian rhythmicity, we established septic mouse models at ZT0 and ZT12. Histopathological analysis showed that liver injury was significantly more severe in mice subjected to LPS challenge at ZT12 than in those challenged at ZT0 (Fig. S1A). Consistent with these findings, serum liver function indices indicated greater hepatic dysfunction at ZT12 (Fig. S1B, C). Inflammatory cytokine analysis showed that the levels of the pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α were significantly lower at ZT0 than at ZT12 (Fig. S1D–F). Moreover, PCR analysis revealed that hepatic BMAL1 and CLOCK expression was significantly higher at ZT0 than at ZT12 (Fig. S1G,H). Collectively, these findings indicate that the severity of sepsis-associated liver injury varies according to the circadian phase and suggest that activation of the BMAL1/CLOCK axis may play an important protective role in this process.

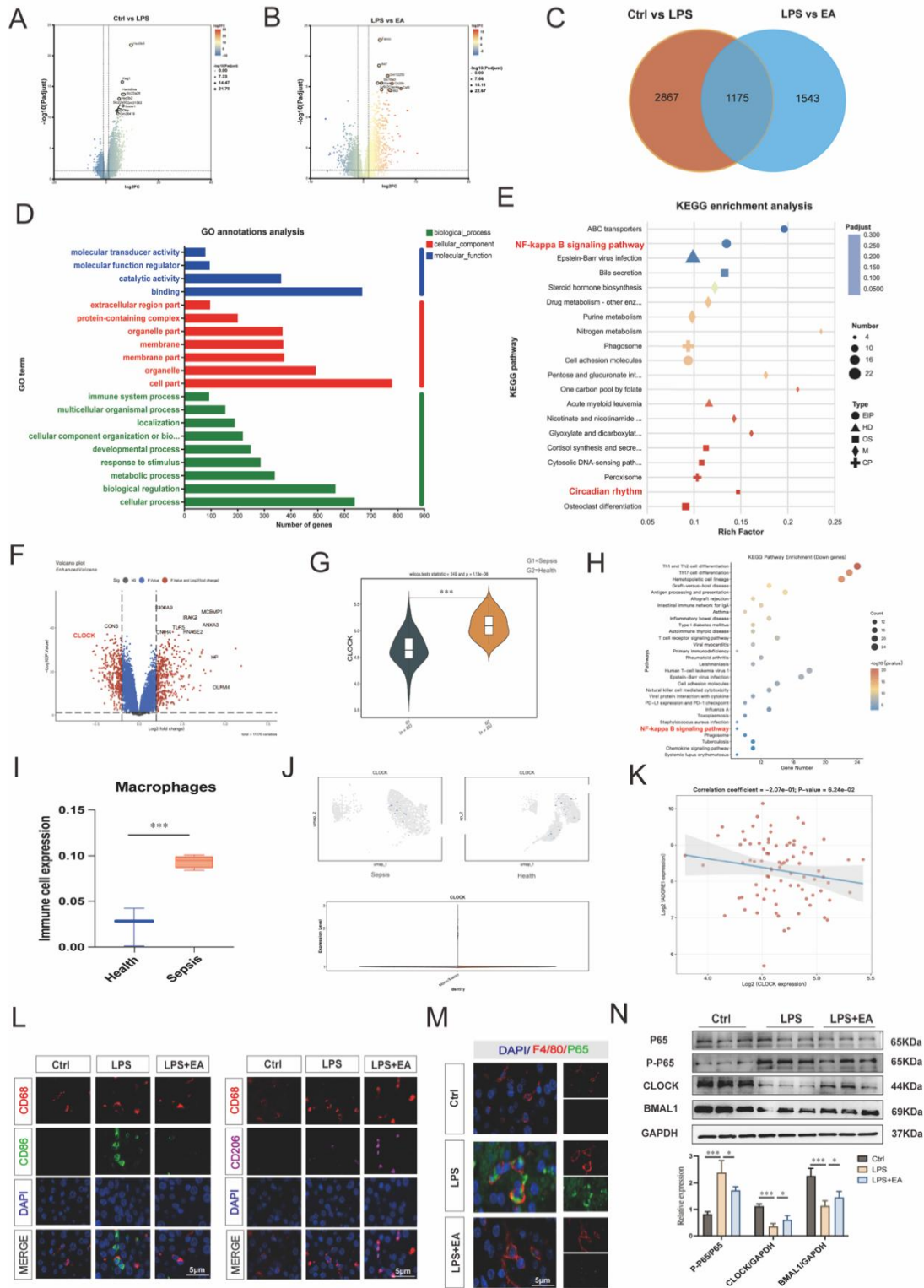


Figure 2. Transcriptomic analysis of the protective effects of EA against SLI. (A, B) Volcano plots (n=6); (C) Venn diagram; (D) Gene Ontology enrichment; (E) KEGG pathway enrichment analysis; (F) Volcano plot ; (G) CLOCK expression between healthy controls and patients with sepsis; (H) KEGG pathway enrichment analysis; (I) ImmuCellAI-based analysis of macrophage infiltration; (J) Single-cell RNA sequencing analysis of CLOCK expression in macrophages; (K) Correlation between CLOCK expression and macrophage activation signatures; (L, M) Immunofluorescence analysis of M1/M2

macrophage polarization and NF- κ B p65 activation (n=4); (N) Western blot analysis(n=4).

EA modulates the gut microbiota composition in septic mice.

A growing body of evidence indicates that intestinal barrier disruption contributes to the development of SLI(Shahid et al., 2024). Morphological staining showed that EA reduced inflammatory cell infiltration in intestinal tissues, promoted the restoration of crypt architecture, and increased the number of goblet cells (Fig. 3A). Immunohistochemical analysis further demonstrated that EA enhanced the expression of ZO-1, thereby alleviating intestinal barrier damage. Given that gut microbiota dysbiosis is an important pathological factor underlying intestinal barrier injury, fecal samples were collected from septic mice for 16S rRNA sequencing. Non-metric multidimensional scaling (NMDS) analysis revealed clear separation among the groups (Fig. 3C). Alpha-diversity analysis showed that EA increased the Shannon index but decreased the Simpson index, indicating alterations in microbial richness and diversity. Venn diagram analysis identified a total of 222 shared taxonomic units (Fig. 3F). At the phylum level, the gut microbiota in all groups was predominantly composed of *Bacteroidota*, *Firmicutes*, *Proteobacteria*, and *Deferribacterota*, with *Bacteroidota* and *Firmicutes* representing the major dominant phyla. Compared with the Sham group, the LPS group exhibited marked alterations in microbial community structure and greater fluctuations in the relative abundance of dominant phyla. Following EA intervention, the microbial composition of the EA group was partially restored compared with that of the LPS group, and the distribution of dominant phyla became more stable. At the genus level, EA increased the relative abundance of *Lactobacillus* and decreased that of *Bacteroides*. At the species level, the relative abundance of *Lactobacillus vaginalis* (*L. V*) was significantly higher in the EA group than in the LPS group. Correlation analysis showed that the abundance of *L. V* was negatively correlated with the levels of the pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α (Fig. 3J–L). These findings suggest that *L. V* may be a key bacterial species involved in the protective effects of EA against sepsis-associated liver injury.

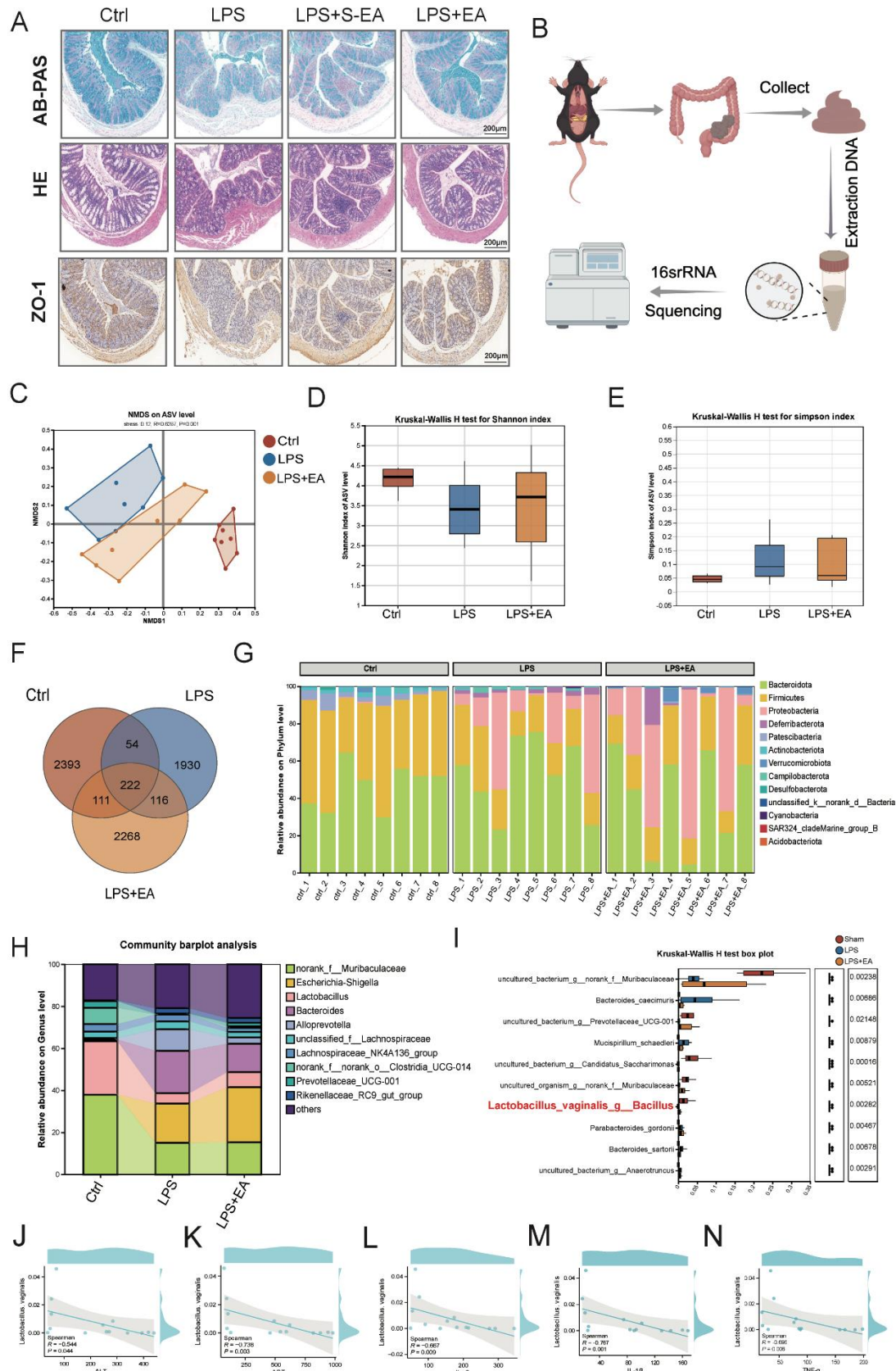


Figure 3. EA modulates the gut microbiota composition in septic mice. (A) Histological staining of colon tissues (n=6); (B) 16S rRNA sequencing workflow (n=6); (C) NMDS analysis (n=6); (D, E) Shannon and Simpson diversity indices (n=6); (F) Venn diagram of OTUs (n=6); (G) Phylum-level microbial

community composition (n=6); (H) Genus-level microbial community composition (n=6); (I) Species-level microbial community composition (n=6); (J–L) Pearson correlation analysis between *L.V* abundance and the levels of pro-inflammatory cytokines.

Gut microbiota remodeling underlies the protective effects of EA against SLI.

To determine whether EA exerts its protective effects against SLI by modulating the gut microbiota, we first established ABX mice and subsequently performed FMT experiments (Fig. 4A). The mortality rate was significantly lower in the FMT-EA group than in the FMT-LPS group (Fig. 4B). Moreover, FMT from EA-treated donor mice reduced the expression of pro-inflammatory cytokines in both the serum and liver tissues of recipient mice (Fig. 4C, D).

Compared with the FMT-LPS group, the FMT-EA group exhibited improved hepatic and renal function, reduced macrophage infiltration, and attenuated liver histopathological injury (Fig. 4E–H). Immunofluorescence staining further showed that FMT-EA decreased the proportion of pro-inflammatory M1 macrophages in the liver and inhibited P65 nuclear translocation in macrophages. Consistently, western blotting revealed that FMT-EA increased hepatic BMAL1 and CLOCK expression while suppressing P65 phosphorylation. Collectively, these findings demonstrate that remodeling of the gut microbiota contributes to the protective effects of EA against SLI.

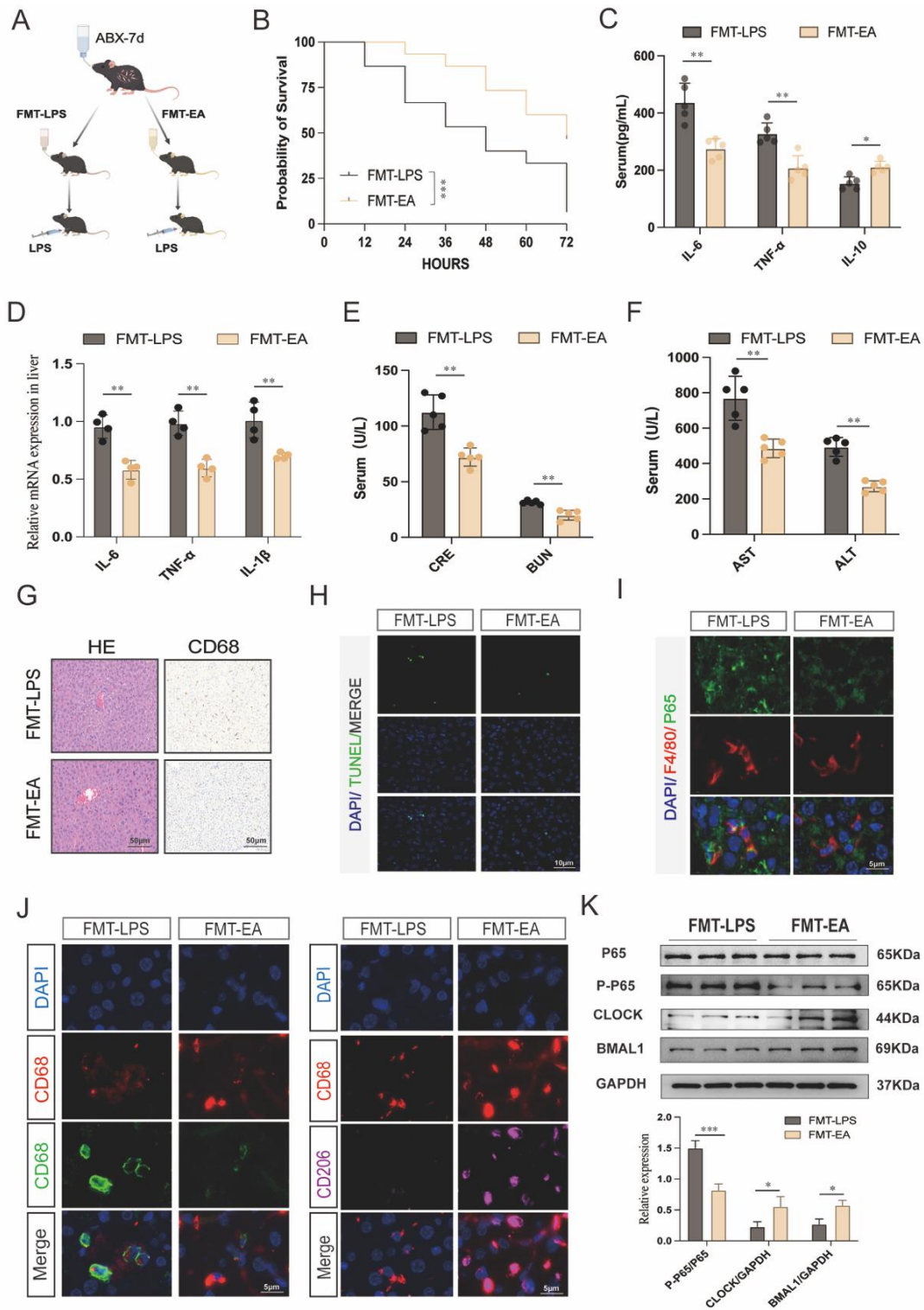


Figure 4. Gut microbiota remodeling underlies the protective effects of EA against SLI. (A) FMT animal experimental; (B) Kaplan–Meier survival curves (n=15); (C) Serum inflammatory cytokine levels (n=5); (D) mRNA analysis of pro-inflammatory cytokine expression in liver tissues (n=4); (E) Serum renal function analysis (n=5); (F) Serum liver function analysis (n=5); (G) Liver H&E and CD68 IHC images (n=4); (H) TUNEL staining of liver tissues (n=4); (I, J) Immunofluorescence analysis of NF- κ B p65 activation and Macrophage polarization (n=4); (K) Western blot analysis (n=4).

***L. V* mediates macrophage polarization by regulating BMAL1/NF- κ B signaling pathway.**

L. V was identified as one of the major differentially abundant bacterial species between the EA and LPS groups. We therefore evaluated its therapeutic potential in SLI through oral administration at a dose of 6×10^8 CFU/day (Fig. 5A). *L. V* treatment not only significantly improved the survival of septic mice (Fig. 5B) but also exerted potent immunomodulatory effects on circulating cytokine production (Fig. 5C). Serum biochemical analyses further showed that *L. V* ameliorated hepatic and renal dysfunction (Fig. 5D, E). Histological and immunohistochemical analyses demonstrated that *L. V* reduced inflammatory cell infiltration and macrophage accumulation in the liver, thereby attenuating hepatic injury (Fig. 5F, G). Moreover, Immunofluorescence staining and Western blotting showed that *L. V* regulated the BMAL1/NF- κ B signaling axis in macrophages and suppressed M1 polarization (Fig. 5H–K). Collectively, these findings suggest that *L. V* alleviates SLI primarily by regulating macrophage polarization, potentially through the BMAL1/NF- κ B signaling pathway.

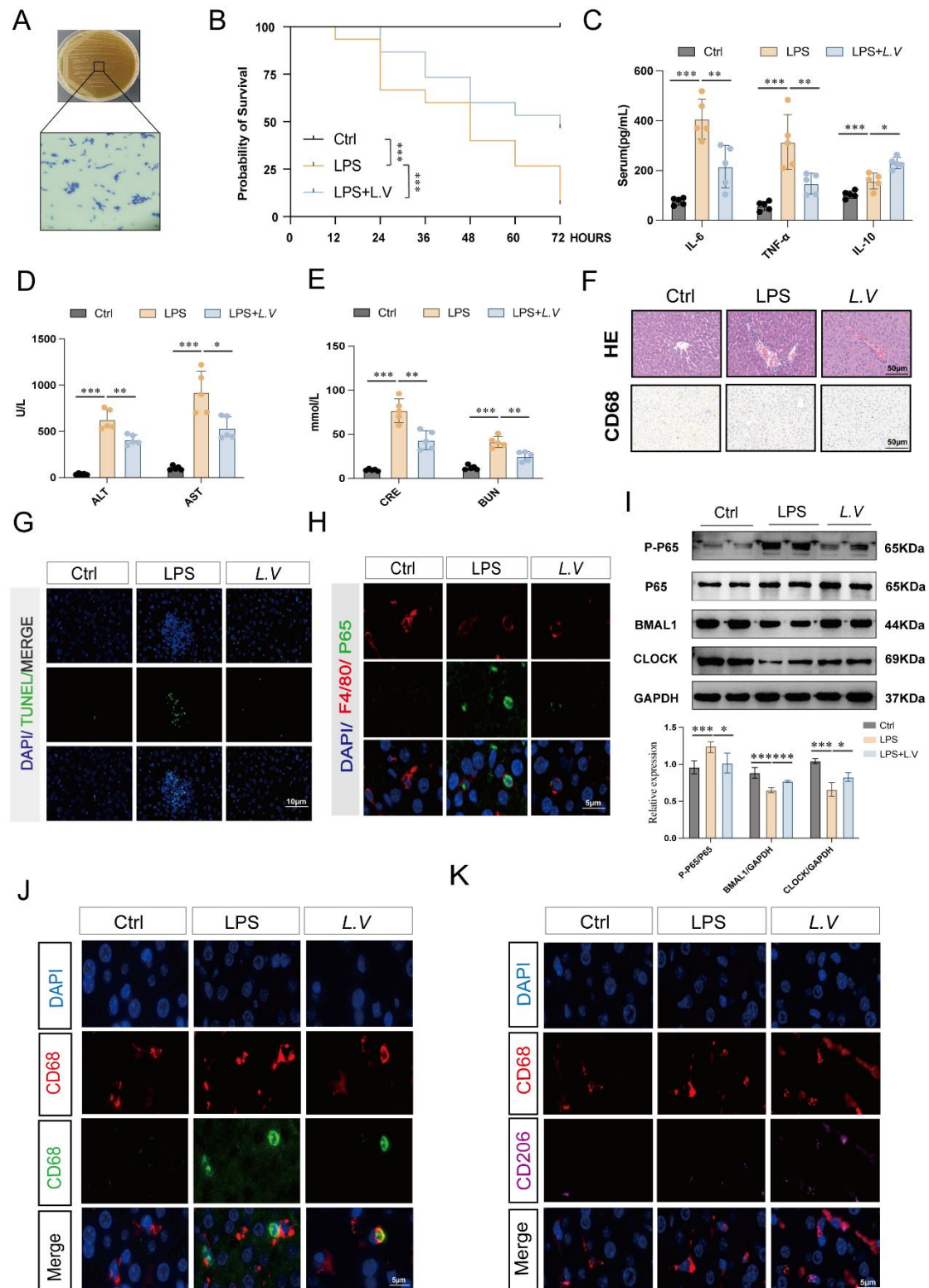
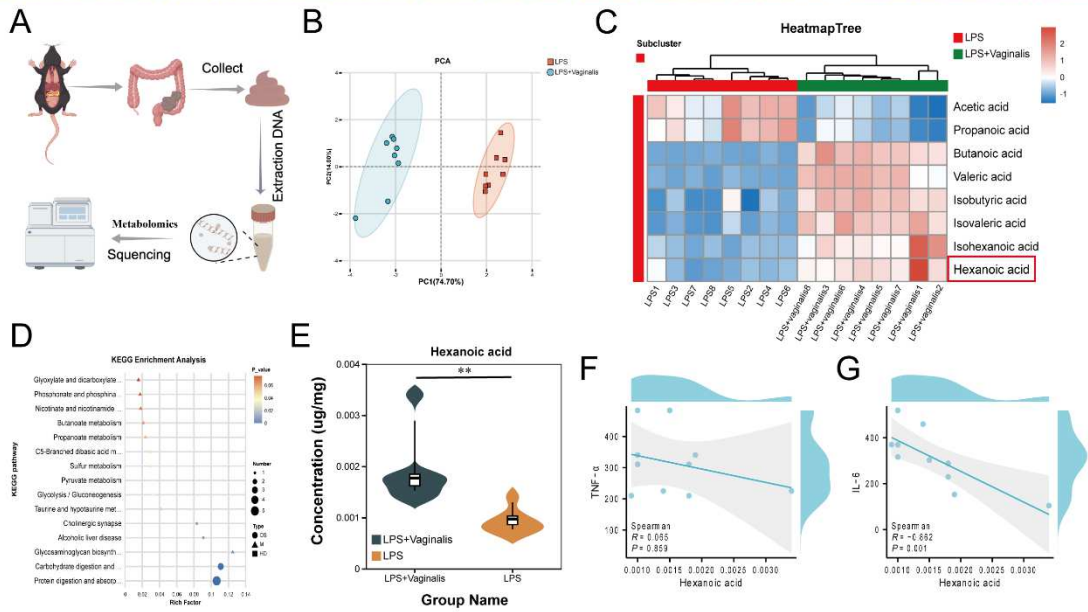


Figure 5. *L. V* alleviates SLI by regulating macrophage polarization through the BMAL1/NF- κ B signaling pathway. (A) Colony morphology of *L. V*; (B) Kaplan–Meier survival curves (n=15); (C) Serum levels of IL-6, TNF- α , and IL-10 (n=5); (D) Serum liver function analysis (n=5); (E) Serum renal function analysis (n=5); (F) Liver H&E and CD68 IHC images (n=4); (G) TUNEL staining of liver tissues (n=4); (H) Immunofluorescence staining of F4/80 and NF- κ B p65 in macrophages (n=4); (I) Western blot analysis (n=4); (J, K) Immunofluorescence analysis macrophage polarization (n=4).

***L. V* alleviates SLI through gut-derived HA.**

Accumulating evidence suggests that gut microbiota-derived metabolites are important mediators regulating host immune responses (Mann et al., 2024). In this study, we performed targeted metabolomic analysis to characterize the alterations in fecal SCFAs between the *L. V* and LPS groups (Fig. 6A). Principal component analysis (PCA) revealed clear separation among the groups (Fig. 6B). Heatmap analysis showed that several SCFAs, including HA (HA), butyric acid, and valeric acid, were elevated in the *L. V* group (Fig. 6C). KEGG enrichment analysis indicated that *L. V* may exert its protective effects against SLI by regulating multiple metabolic pathways, including glyoxylate and dicarboxylate metabolism, phosphonate and phosphinate metabolism, and nicotinate and nicotinamide metabolism (Fig. 6D). Previous studies have shown that HA possesses anti-inflammatory and antioxidant properties. Consistent with this, our results demonstrated that *L. V* markedly increased the fecal level of HA in septic mice (Fig. 6E). Correlation analysis further showed that HA levels were significantly negatively correlated with the expression of pro-inflammatory cytokines (Fig. 6F, G). To further investigate the relationship between SCFAs alterations and the inflammatory storm in sepsis, we collected serum samples from patients with sepsis and healthy controls (Fig. 6H). The results showed that the levels of pro-inflammatory cytokines were significantly elevated in the sera of patients with sepsis (Fig. 6I–K). Targeted metabolomic analysis revealed a marked difference in metabolic profiles between patients with sepsis and healthy controls (Fig. 6L). Volcano plot and differential heatmap analyses further demonstrated widespread dysregulation of SCFAs metabolism in sepsis, among which HA was significantly decreased in patients with sepsis (Fig. 6M–O). Pearson correlation analysis showed that HA levels were significantly negatively correlated with the expression of the pro-inflammatory cytokines IL-6, IL-1 β , and TNF- α (Fig. 6P–R). Collectively, these findings indicate that SCFAs metabolism is severely disturbed in sepsis, and that reduced HA levels may contribute to disease progression.

Sequencing of Mouse samples



Sequencing of People Samples

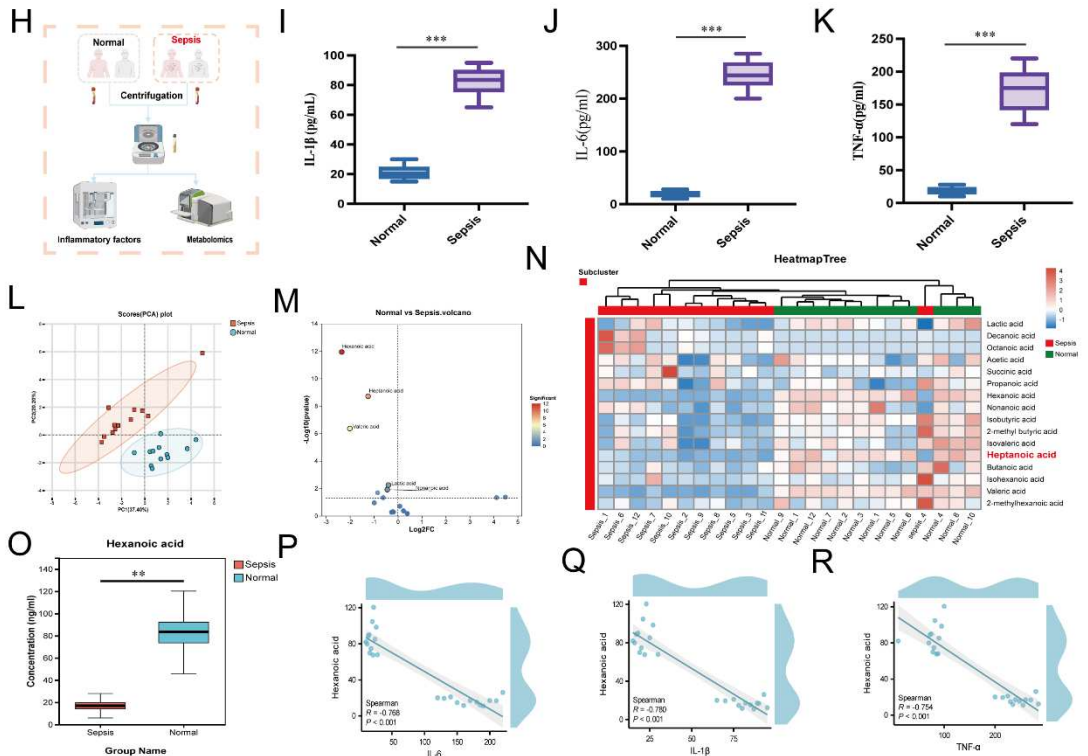


Figure 6. *L. V* alleviates SLI through HA. (A) Fecal sample collection; (B) PCA of metabolic profiles; (C) Heatmap showing differential SCFAs levels (n=8); (D) KEGG pathway enrichment analysis; (E) Fecal HA levels (n=8); (F, G) Spearman correlation analyses between fecal HA levels and TNF- α and IL-6 levels, respectively. (H) Human serum sample collection; (I–K) Serum levels of IL-1 β , IL-6, and TNF- α (n=12); (L) PCA of serum metabolic profiles (n=12); (M) Volcano map; (N) Heatmap of SCFAs; (O) Serum HA levels (n=12); (P–R) Spearman correlation analyses between serum HA levels and IL-6, IL-

1 β , and TNF- α levels, respectively.

HA regulates macrophage polarization and alleviates SLI.

To investigate the effects of HA on SLI, mice were orally administered sodium hexanoate for 3 days before model induction (Fig. 7A). The results showed that HA significantly improved the survival of septic mice (Fig. 7B). Meanwhile, ELISA and biochemical analyses demonstrated that sodium hexanoate suppressed the expression of pro-inflammatory cytokines and improved hepatic and renal function (Fig. 7C–F). H&E staining and immunohistochemical analysis further showed that sodium hexanoate alleviated liver injury and reduced macrophage infiltration in liver tissues (Fig. 7G, H). In addition, sodium hexanoate decreased the proportion of M1 macrophages while increasing the proportion of M2 macrophages, and immunofluorescence staining further confirmed that HA inhibited NF- κ B p65 activation in macrophages (Fig. 7I–K). Finally, western blot analysis verified that sodium hexanoate inhibited P-P65 expression while increasing the expression of BMAL1 and CLOCK in liver tissues (Fig. 7L).

To determine the role of BMAL1 in SLI and whether it mediates the hepatoprotective effects of sodium hexanoate, mice were intraperitoneally administered the BMAL1 inhibitor STL1267. Pharmacological inhibition of BMAL1 further reduced the survival of septic mice. Notably, STL1267 markedly attenuated the survival benefit conferred by sodium hexanoate (Fig. 7B). Consistent with the survival findings, liver function and histopathological analyses showed that STL1267 aggravated hepatic dysfunction and liver tissue injury and partially reversed the protective effects of sodium hexanoate (Fig. 7C–H). Moreover, sodium hexanoate inhibited hepatic macrophage polarization toward the pro-inflammatory M1 phenotype and suppressed NF- κ B signaling activation, whereas STL1267 markedly weakened these effects, resulting in increased M1 macrophage polarization and reactivation of the NF- κ B pathway (Fig. 7I–M). Collectively, these findings indicate that BMAL1 plays an important protective role in sepsis-associated liver injury and suggest that sodium hexanoate alleviates liver injury by activating BMAL1, suppressing NF- κ B signaling, and consequently regulating macrophage polarization.

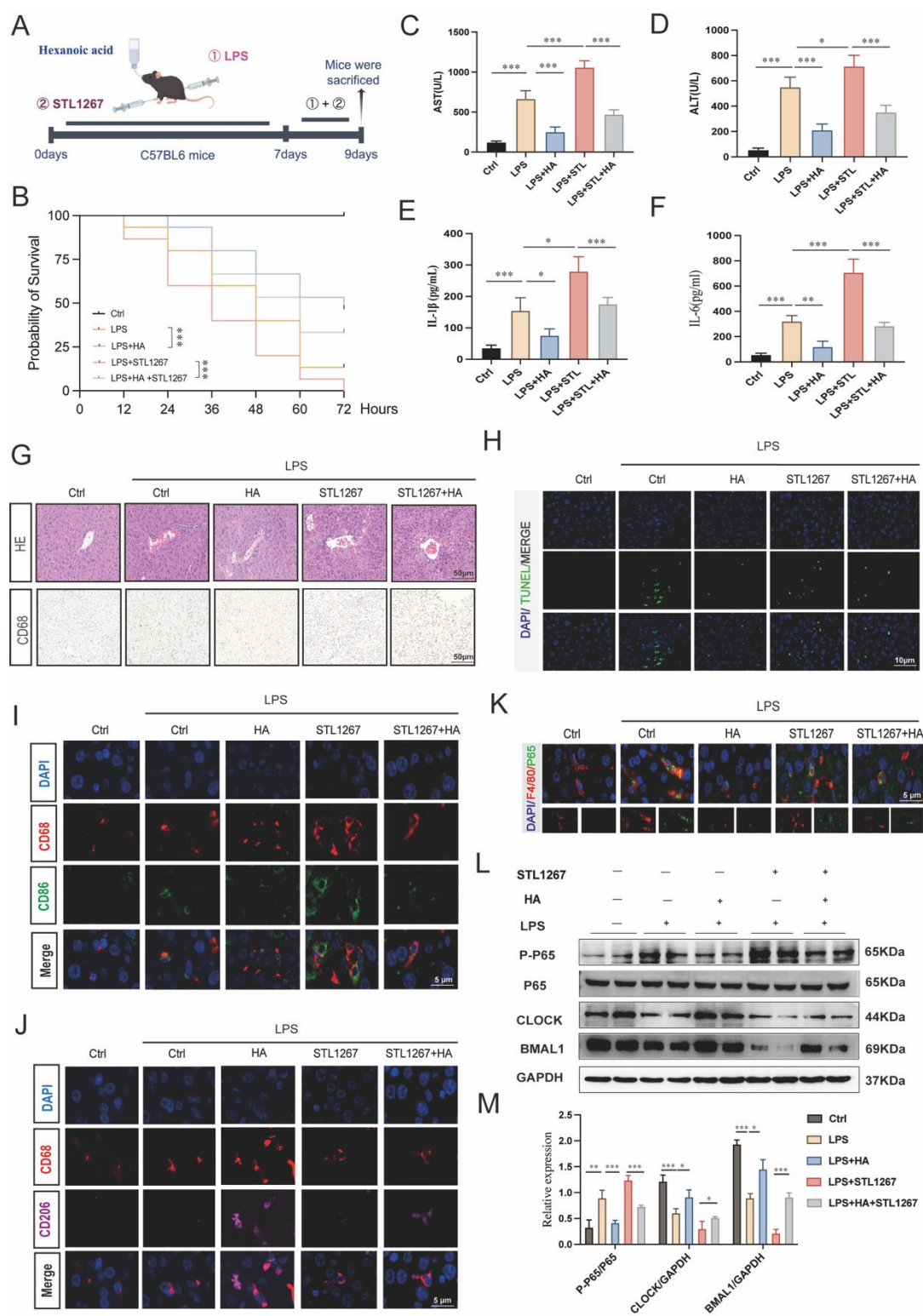


Figure 7. HA alleviates SLI by activating BMAL1 and suppressing NF- κ B signaling. (A) Experimental design C57BL/6 mice. (B) Kaplan–Meier survival curves (n=15). (C, D) Serum liver function analysis (n=5). (E, F) Serum IL-1 β and IL-6 levels (n=5). (G) Liver H&E and CD68 IHC images (n=4). (H) TUNEL staining of liver tissues (n=4). (I, J) Immunofluorescence analysis macrophage polarization (n=4). (K) Immunofluorescence staining of F4/80 and NF- κ B p65 in macrophages (n=4). (L, M) Western blot analysis (n=4).

HA targets BMAL1 to inhibit M1 macrophage polarization.

To further validate the protective effects of HA, we performed a series of in vitro experiments (Fig. 8A). CCK-8 assays showed that HA at concentrations of 0.1, 0.25, and 0.5 mM had no significant effect on RAW264.7 cell viability, whereas moderate inhibition of cell viability was observed at 1 mM (Fig. 8B). Further experiments demonstrated that HA suppressed LPS-induced expression and release of pro-inflammatory cytokines in macrophages (Fig. 8C-E) and attenuated AML12 cell apoptosis (Fig. 8F) induced by RAW264.7 cell-conditioned medium, indicating its anti-inflammatory and hepatoprotective effects. Moreover, HA promoted macrophage polarization toward the M2 phenotype (Fig. 8G). WB and IF analyses showed that HA markedly increased BMAL1 and CLOCK expression in macrophages while inhibiting activation of the NF- κ B signaling pathway (Fig. 8H, I). In vitro treatment with the BMAL1 inhibitor STL1267 partially reversed the regulatory effects of HA on inflammatory responses, macrophage polarization, and NF- κ B activation, consistent with the in vivo findings.

To investigate whether HA regulates the NF- κ B signaling pathway by directly targeting BMAL1, molecular docking, molecular dynamics (MD) simulations, and SPR assays were performed. Molecular docking analysis showed that HA bound to BMAL1 with a binding energy of -5.736 kcal/mol, indicating a potential interaction between HA and BMAL1 (Fig. 8J). Specifically, HA formed a hydrogen bond with ASN438 and hydrophobic interactions with TRP420, ILE434, and HIS344 (Fig. 8K). The 100-ns MD simulation demonstrated that the HA-BMAL1 complex maintained a stable conformation, as indicated by the relatively small fluctuations in the RMSD values of the complex and ligand (Fig. S2A, B), the nearly constant radius of gyration and solvent-accessible surface area (Fig. S2C, D), and the moderate residue-level RMSF values (Fig. S2F). Dynamic hydrogen-bond interactions between HA and residues within the BMAL1-binding pocket were maintained throughout the simulation, contributing to ligand stabilization (Fig. S2E). Furthermore, MM/PBSA analysis yielded a binding free energy of -25.12 kcal/mol, indicating favorable thermodynamic stability of the HA-BMAL1 complex (Fig. S2G). SPR analysis confirmed a direct interaction between HA and BMAL1, with the response signal gradually increasing as the HA concentration increased. Kinetic fitting yielded an equilibrium dissociation constant (K_D) of 2.056×10^{-6} M, indicating that they had a high binding affinity. Collectively, these findings demonstrate that HA directly binds to BMAL1 and suggest that HA may inhibit M1 macrophage polarization by targeting BMAL1 and suppressing the NF- κ B signaling pathway.

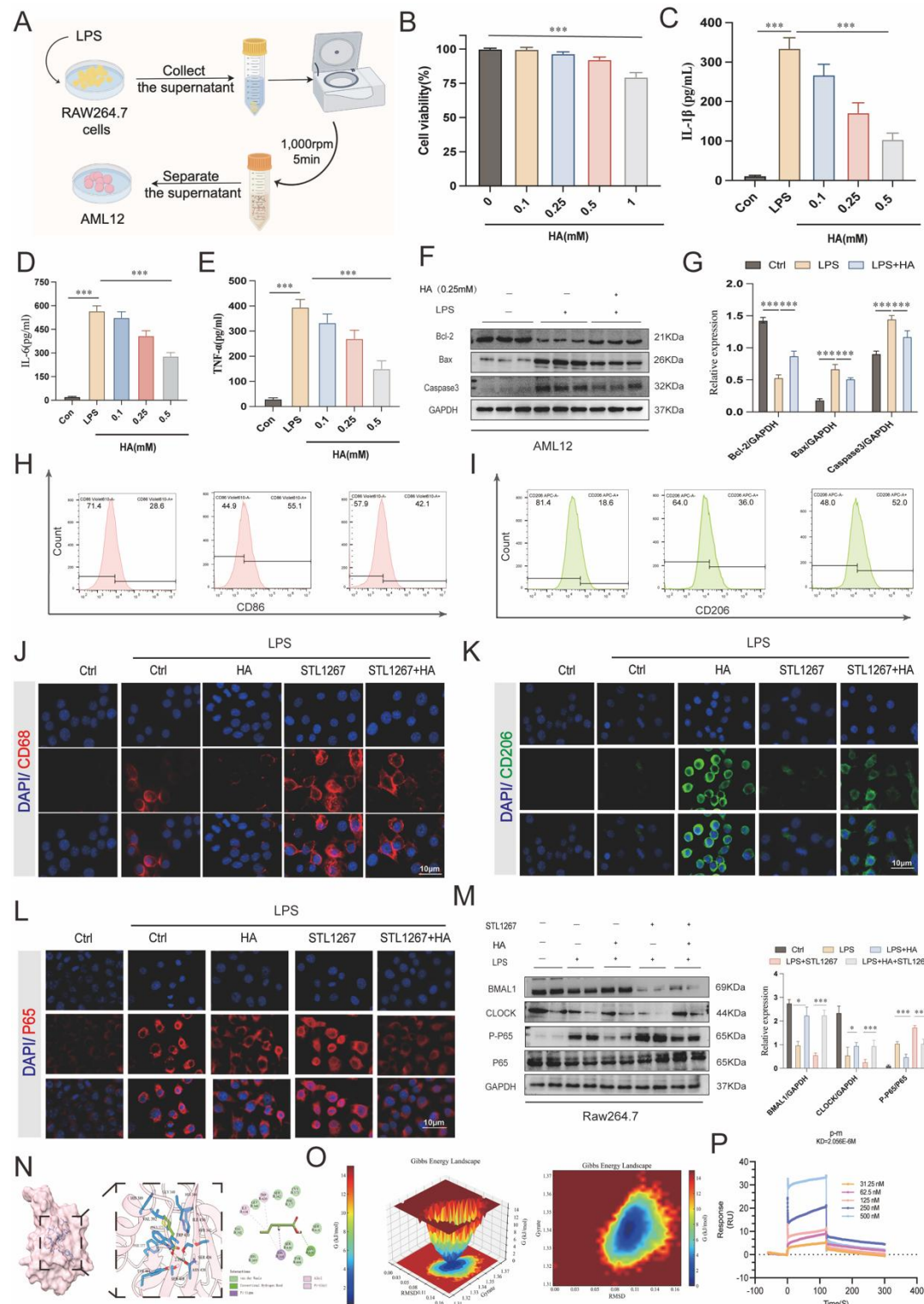


Figure 8 HA suppresses M1 macrophage polarization by targeting the BMAL1/NF-κB signaling pathway. (A) Vitro experimental procedure; (B) CCK-8 assay of cell viability (n=5); (C-E) Levels of pro-inflammatory cytokines in macrophage culture supernatants (n=5); (F) Expression of apoptosis-related proteins in hepatocytes (n=4); (G) Flow cytometric analysis of macrophage polarization (n=4); (H-I) Immunofluorescence analysis macrophage polarization (n=4); (J) Immunofluorescence staining of F4/80 and NF-κB p65 in macrophages (n=4); (K) Western blot analysis (n=4).

Preparation, characterization, and targeting evaluation of HA@L-INT

To overcome the short in vivo half-life of free HA and its limited accumulation in inflamed hepatic tissue, we developed an engineered HA@L-INT (Fig. 9A). Transmission electron microscopy revealed that both HA@L and HA@L-INT exhibited uniform, well-defined spherical morphologies (Fig. 9B). Based on the UV-visible spectrophotometric calibration curve for sodium hexanoate (Fig. S3), the drug-loading capacity and encapsulation efficiency were calculated to be 6.88% and 84.33%, respectively. Dynamic light scattering analysis showed that conjugation with INT-777 slightly increased the hydrodynamic diameter of the liposomes from 169 to 176 nm, while the polydispersity index remained below 0.2, indicating a narrow size distribution and no appreciable aggregation following surface functionalization (Fig. 9C, D). Moreover, the zeta potential decreased from +12.2 mV for HA@L to +5.8 mV for HA@L-INT (Fig. 9E), confirming successful surface modification.

To evaluate its pH responsiveness and endosomal escape potential, HA@L-INT was incubated at pH 5.0 to mimic the endosomal microenvironment. Acidification protonated cholesteryl hemisuccinate, exposing the cationic properties of DOTAP and increasing the zeta potential to above +25 mV, while the particle size increased to over 210 nm (Fig. 9G). This acid-triggered charge reversal and DOPE-mediated membrane reorganization may destabilize the endosomal membrane, thereby facilitating endosomal escape and cytosolic HA release. The cellular uptake and biocompatibility of HA@L-INT were subsequently evaluated. Confocal laser scanning microscopy revealed that FITC-labeled HA@L-INT was efficiently internalized by RAW264.7, as evidenced by strong intracellular fluorescence distributed throughout the cytoplasm and perinuclear regions (Fig. 9F). This enhanced cellular uptake may be attributed to the affinity of INT-777 for TGR5 expressed on macrophages. Calcein-AM/propidium iodide dual staining further showed predominantly viable cells with minimal PI-positive staining following HA@L-INT treatment, indicating negligible acute cytotoxicity and favorable cytocompatibility toward RAW264.7 macrophages (Fig. 9H). Consistently, the low hemolysis rate observed in the hemolysis assay supported the favorable hemocompatibility of HA@L-INT (Fig. S4). Collectively, these findings demonstrate that HA@L-INT possesses efficient macrophage uptake and a favorable preliminary biosafety profile. In vivo biodistribution analysis in LPS-induced septic mice showed that HA@L-INT achieved greater and more sustained hepatic accumulation than non-targeted HA@L, with liver fluorescence peaking at 6–9 h after intravenous administration (Fig. 9I). This enhanced accumulation may be attributed to INT-777-mediated recognition and uptake by TGR5-expressing hepatic macrophages, supporting HA@L-INT as a targeted delivery platform for SLI.

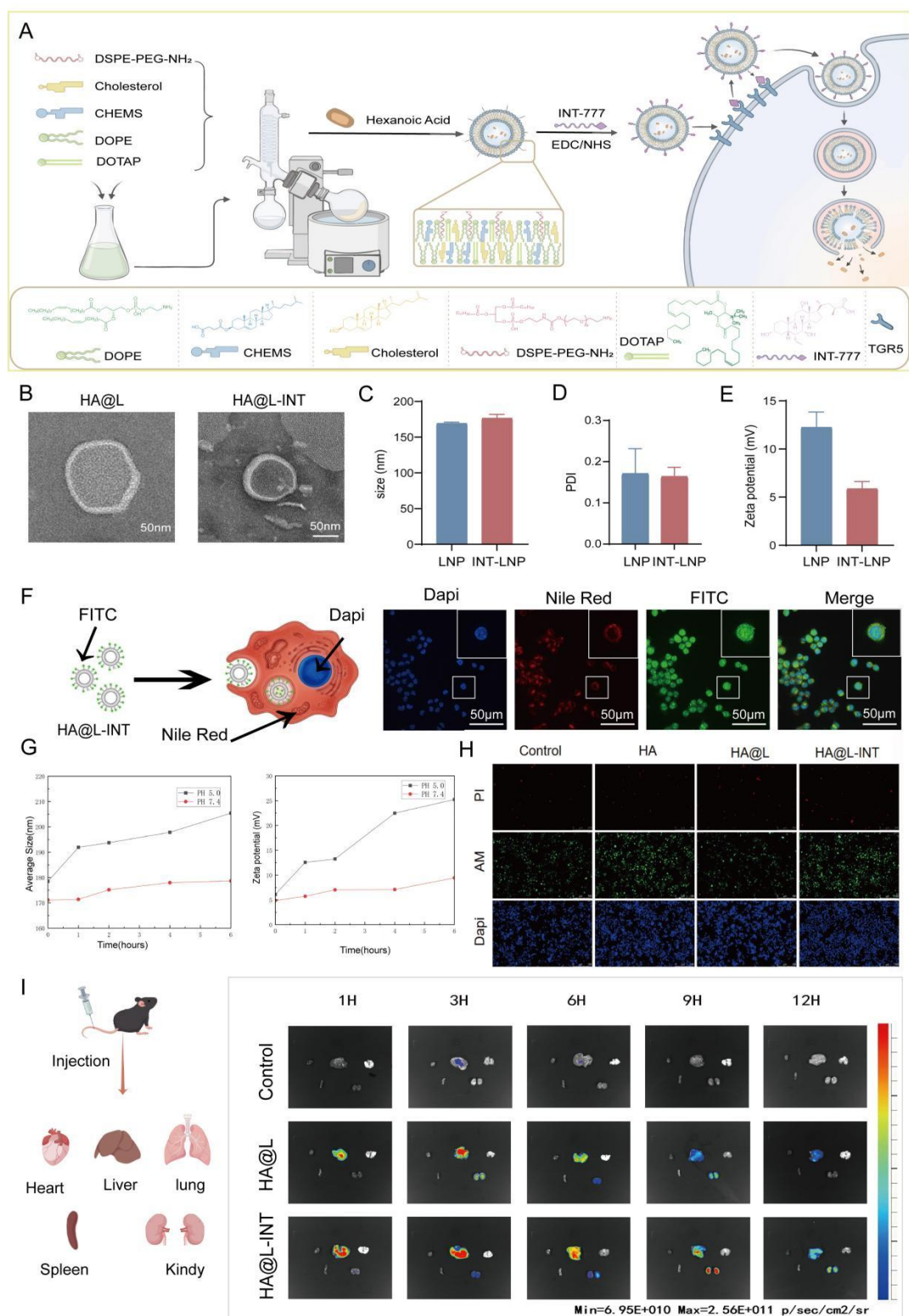


Figure 9. Physicochemical characterization and biological evaluation of HA@L-INT. (A) Schematic illustration of HA@L-INT preparation. (B) TEM images of HA@L and HA@L-INT. Scale bar = 50 nm. (C-E) Hydrodynamic size (C), PDI (D), and surface Zeta potential (E) Profiles (n=3). (F) CLSM images of RAW264.7 cells incubated with FITC-HA@L-INT. Blue: DAPI; Red: Nile Red; Green: FITC. Scale bar = 50 μm. (G) Time-dependent variations of size and Zeta potential in physiological (pH 7.4) and acidic (pH 5.0) conditions. (H) Live/Dead (Calcein-AM/PI) staining of RAW264.7 cells post-treatments.

(I) Ex vivo fluorescence imaging of major organs from septic mice at indicated time points post-injection.

HA@L-INT exhibits superior therapeutic efficacy against SLI compared with free HA.

In the septic mouse model, HA@L-INT exhibited superior therapeutic efficacy compared with free HA, as evidenced by markedly attenuated hepatic histopathological injury, reduced serum AST, ALT, IL-6, and IL-1 β levels (Fig. 10A–E), and suppressed the expression of pro-inflammatory cytokines in liver tissues (Fig. 10F). Furthermore, immunofluorescence staining and Western blotting demonstrated that HA@L-INT activated BMAL1 and subsequently suppressed NF- κ B signaling, thereby promoting macrophage polarization toward the anti-inflammatory M2 phenotype. These findings indicate that HA@L-INT exhibits superior hepatoprotective and anti-inflammatory effects compared with free HA. To evaluate the biosafety of HA@L-INT, hematological parameters and histopathological changes in major organs were assessed. No significant differences were observed in any of the hematological indices among the Ctrl, @L, and @L-INT groups (Fig. 10K–N). Moreover, H&E staining revealed preserved tissue architecture in the heart, liver, spleen, lung, kidney, and colon, with no apparent inflammatory infiltration, necrosis, edema, or other pathological abnormalities (Fig. 10O). Collectively, these findings demonstrate that HA@L-INT exhibits favorable systemic biocompatibility and biosafety in vivo.

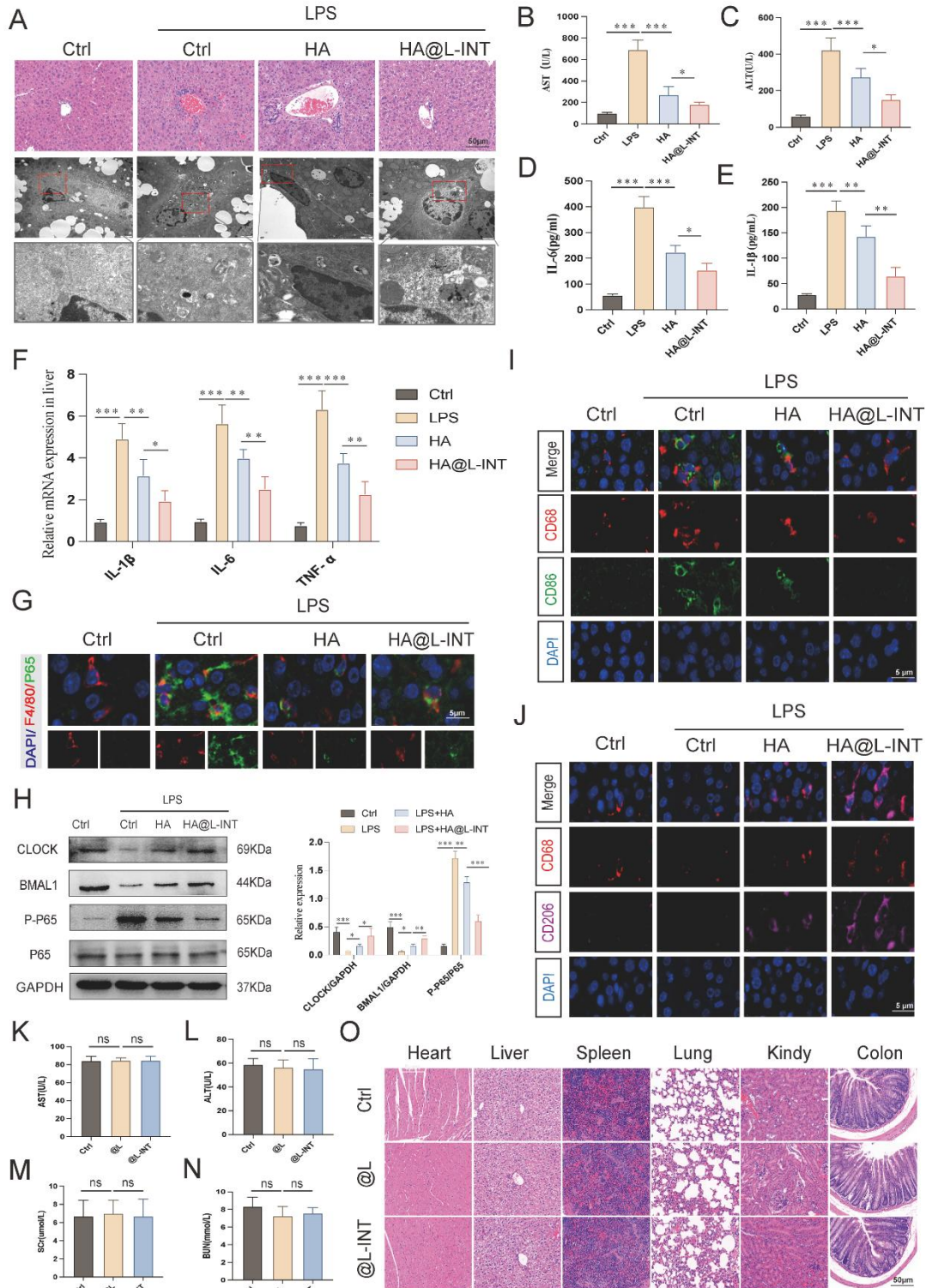


Figure 10 Protective effects of modified HA@L-INT in SLI. (A) H&E staining and TEM images of liver tissue(n=5). (B,C) Serum liver function(n=5); (D,E) Serum cytokines(n=5); (F) Hepatic mRNA expression of inflammatory cytokines(n=4); (G) Immunofluorescence staining of F4/80 and NF-κB p65 in macrophages(n=4); (H)Western blot analysis(n=4); (I,J)Immunofluorescence analysis macrophage polarization (n=4); (K,L) Serum liver function(n=4); (M,N) Serum kidney function(n=4); (O) H&E staining(n=4).

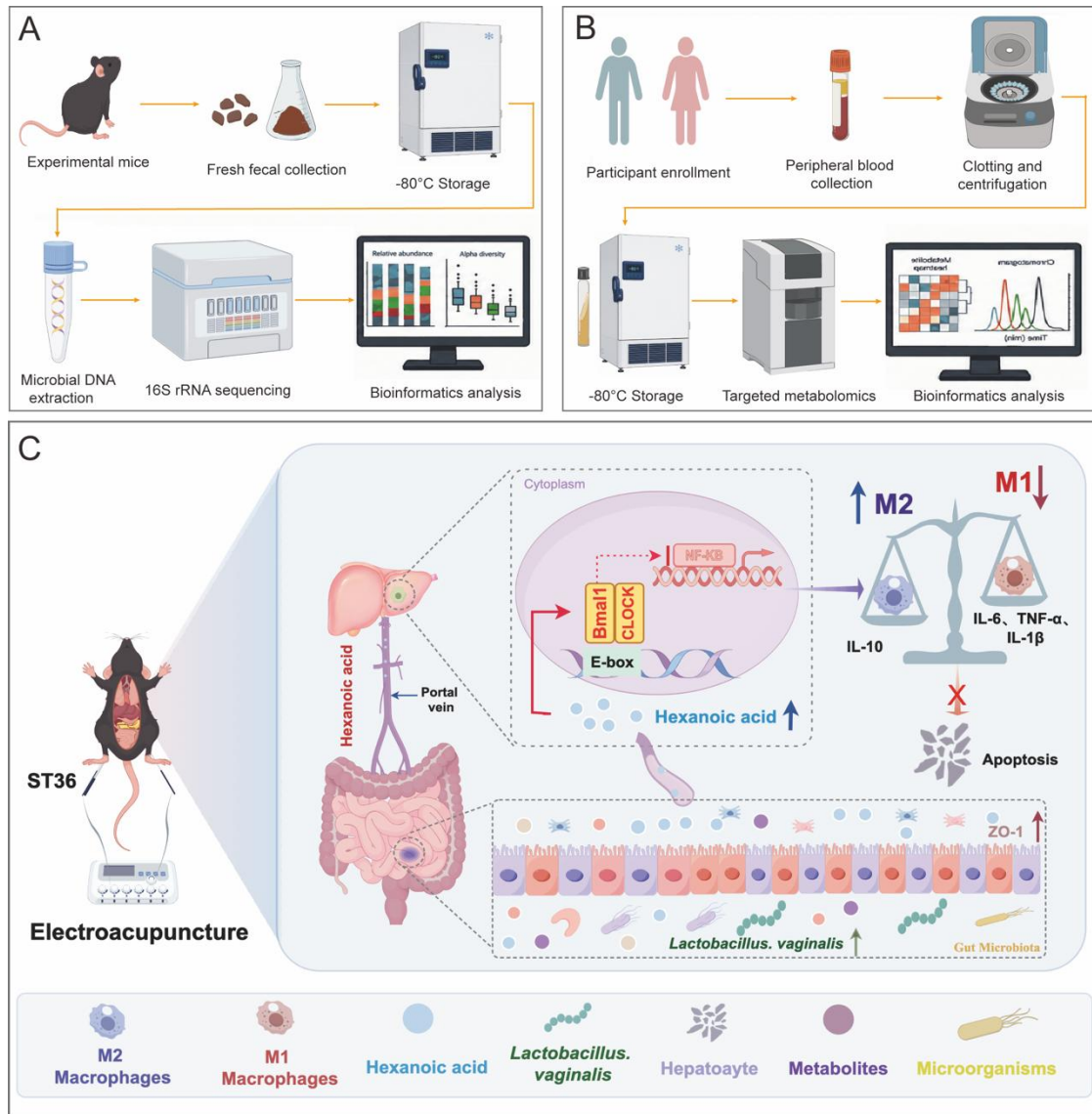


Figure 11 Proposed mechanism of EA in SLI

Discussion

SLI is a severe complication of sepsis and is closely associated with poor outcomes in ICU patients; however, effective therapeutic strategies remain lacking (Wang et al., 2025a). Previous studies have demonstrated that sepsis-induced disruption of metabolic homeostasis is an important pathological factor contributing to the development and progression of liver injury (Van Wyngene et al., 2020; Zhang et al., 2024). In this study, we found that HA levels were significantly reduced in patients with sepsis. Electroacupuncture enriched *L. vaginalis* and promoted the production of gut-derived HA, thereby suppressing M1 macrophage polarization and alleviating SLI (Fig. 11).

The development and progression of SLI involve multiple pathological processes, including dysregulated systemic inflammation, hepatic microcirculatory dysfunction, disrupted metabolic homeostasis, and impairment of the gut microbial dysbiosis (Beyer et al., 2022). Increasing evidence indicates that disruption of gut microbial homeostasis is an important pathological factor contributing to the initiation and progression of liver injury (Elias et al., 2023; Zhang et al., 2022). During sepsis,

systemic inflammation and intestinal hypoperfusion disrupt epithelial tight junctions, facilitating the translocation of gut-derived bacteria and their metabolites to the liver through the portal vein and mesenteric lymphatic circulation, thereby aggravating hepatic injury(Potruch et al., 2022; Strnad et al., 2017). In the present study, EA markedly alleviated sepsis-induced intestinal barrier damage. Moreover, 16S rRNA sequencing showed that EA reshaped the gut microbial community in septic mice, as evidenced particularly by an increased relative abundance of *Lactobacillus*. Previous research demonstrated that *Lactobacillus plantarum* Lp2 attenuated LPS-induced acute liver injury by suppressing TLR4/NF- κ B signaling(Chen et al., 2022). Notably, our species-level analysis revealed that EA significantly increased the relative abundance of *L. V. L. V*. *V* is a Gram-positive, facultatively anaerobic, lactic acid-producing bacterium(Puglisi et al., 2019). It has been reported to alleviate DSS-induced colitis by modulating the gut microbiota, increasing the production of 3-indoleacrylic acid, and restoring the Treg/Th17 balance(Wang et al., 2025b). Furthermore, *L. V*-derived β -galactosidase converts dietary daidzin into daidzein, which attenuates acetaminophen-induced liver injury by suppressing Fdps-mediated hepatocyte ferroptosis through the AKT/Nrf2 axis(Zeng et al., 2023). Fecal microbiota transplantation further demonstrated that gut microbiota derived from EA-treated mice improved survival, attenuated systemic and hepatic inflammation, and ameliorated hepatic as well as histopathological liver injury in septic recipient mice. Collectively, these findings suggest that EA protects against SLI, at least in part, by restoring gut microbial homeostasis.

Growing evidence suggests that gut microbiota-derived metabolites regulate hepatic metabolic and immune homeostasis through the gut–liver axis, thereby influencing the development and progression of SLI (Hu et al., 2025; Strnad et al., 2017). Among these metabolites, bile acid derivatives, fatty acids, and amino acid-related metabolites play important roles(Agus et al., 2021). HA is a gut microbiota-derived fatty acid metabolite with potential immunomodulatory and anti-inflammatory properties(Fitzgerald et al., 2025). Previous studies have shown that HA suppresses the production and release of inflammatory mediators, including IL-32, IL-18, and IL-1 β , from LPS-stimulated monocytes(El-Far et al., 2021). In the present study, targeted metabolomic analysis revealed that HA levels were significantly decreased in both patients and mice with sepsis and were negatively correlated with circulating IL-1 β , TNF- α , and IL-6 levels. EA restored HA production in septic mice and exerted protective effects against SLI.

The circadian system maintains internal homeostasis by coordinating metabolic, immune, and inflammatory responses(Bass and Lazar, 2016). As a core circadian clock protein, BMAL1 plays an essential role in maintaining hepatic rhythmicity and metabolic homeostasis and in regulating immune responses(Jacobi et al., 2015). BMAL1 deficiency impairs NRF2-mediated antioxidant responses and disrupts mitochondrial homeostasis, resulting in ROS accumulation and HIF-1 α stabilization, which subsequently promotes IL-1 β production (Early et al., 2018). Myeloid-specific BMAL1 deficiency enhances NF- κ B activity and miR-155 induction, thereby amplifying LPS-induced inflammatory responses and increasing susceptibility to endotoxemia in mice(Curtis et al., 2015). Li et al. reported that BMAL1 expression was markedly reduced in macrophages and hepatic Kupffer cells during sepsis, whereas restoration of BMAL1 expression suppressed pro-inflammatory cytokine production and alleviated sepsis-associated organ injury(Li et al., 2025). In the present study, EA restored hepatic

BMAL1 expression and inhibited NF- κ B signaling activation in septic mice, with similar effects observed following HA administration. SPR analysis further demonstrated a direct interaction between HA and BMAL1. Conversely, the BMAL1 inhibitor STL1267 attenuated the inhibitory effects of HA on NF- κ B activation and macrophage M1 polarization and partially reversed its hepatoprotective effects. Collectively, these findings suggest that EA alleviates SLI by promoting the production of *L.V*-derived HA and subsequently regulating the BMAL1/NF- κ B signaling pathway.

Although HA exhibits potential hepatoprotective and immunomodulatory activities, it undergoes rapid oxidative metabolism in vivo, making it difficult to maintain effective systemic exposure (Goetzman et al., 2020; Schönfeld and Wojtczak, 2016). Moreover, its lack of selective delivery to inflamed liver tissue and hepatic macrophages limits its therapeutic efficacy and translational potential. To address these limitations, we designed and developed a pH-responsive liposomal delivery system to improve the stability of HA, prolong its systemic circulation, and facilitate its selective release within the acidic inflammatory microenvironment. Given the expression of TGR5 in macrophages, the liposomal surface was further functionalized with the TGR5 agonist INT-777 to enhance nanoparticle targeting of hepatic macrophages, resulting in the successful construction of an INT-777-modified, pH-responsive liposome loaded with HA. Through the synergistic effects of inflammation-responsive release and macrophage-targeted delivery, this system was expected to increase the effective accumulation of HA in injured liver tissue, thereby enhancing its regulatory effects on macrophage polarization and hepatic inflammation. Notably, compared with free HA, HA@L-INT exhibited more pronounced therapeutic effects, more effectively suppressed hepatic inflammation, and improved the in vivo stability and effective exposure of HA. These findings indicate that pH-responsive release combined with INT-777-mediated macrophage targeting can overcome the rapid metabolism and poor tissue selectivity of free HA. Thus, HA@L-INT represents a promising nanodelivery strategy for the treatment of SLI.

In conclusion, this study demonstrates that EA alleviates SLI by restoring gut microbial homeostasis, enriching *L.V*, and promoting the production of gut-derived HA. These findings reveal a previously unrecognized mechanism underlying the therapeutic effects of EA on SLI from the perspective of the gut–liver axis. Furthermore, HA exerted hepatoprotective effects by targeting BMAL1, modulating the BMAL1/NF- κ B signaling pathway, and suppressing macrophage M1 polarization. To overcome the rapid oxidative metabolism, limited duration of effective exposure, and insufficient targeting capacity of free HA, we further developed HA@L-INT, an INT-777-modified, pH-responsive liposomal delivery system. HA@L-INT enhanced the selective delivery and therapeutic efficacy of HA in inflamed liver tissue and hepatic macrophages, providing a promising strategy for the targeted treatment of SLI.

Author Contributions

Shifan Yan and Yu Jiang conceived and designed the study. Zehong Liu and Daocheng Zhu performed the experiments and acquired the data. Jun Xiong and Li Chen analyzed and interpreted the data. Ting Yu drafted the manuscript. Li Chen and Yu Jiang supervised the study and critically revised the manuscript. All authors reviewed and approved the final manuscript.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Clinical trial number: not applicable.

Availability of data and material

Data sets used and/or analyzed during the current study period are available from corresponding authors upon reasonable request.

Consent for publication

All authors critically reviewed the content of the manuscript. The consent for publication was obtained from all authors.

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