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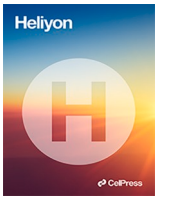

Research article

REV-ERBα negatively regulates NLRP6 transcription and reduces the severity of *Salmonella* infection in mice

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ABSTRACT

Non-typhoidal *Salmonella* infection is among the most frequent foodborne diseases threatening human health worldwide. The host circadian clock orchestrates daily rhythms to adapt to environmental changes, including coordinating immune function in response to potential infections. However, the molecular mechanisms underlying the interplay between the circadian clock and the immune system in modulating infection processes are incompletely understood. Here, we demonstrate that NLRP6, a novel nucleotide-oligomerization domain (NOD)-like receptor (NLR) family member highly expressed in the intestine, is closely associated with the differential day–night response to *Salmonella* infection. The core clock component REV-ERBα negatively regulates NLRP6 transcription, leading to the rhythmic expression of NLRP6 and the secretion of IL-18 in intestinal epithelial cells, playing a crucial role in mediating the differential day–night response to *Salmonella* infection. Activating REV-ERBα with agonist SR9009 in wild-type mice attenuated the severity of infection by decreasing the NLRP6 level in intestinal epithelial cells. Our findings provide new insights into the association between the host circadian clock and the immune response to enteric infections by revealing the regulation of *Salmonella* infection via the inhibitory effect of REV-ERBα on NLRP6 transcription. Targeting REV-ERBα to modulate NLRP6 activation may be a potential therapeutic strategy for bacterial infections.

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1. Introduction

Non-typhoidal *Salmonella* infection is a major cause of foodborne illness worldwide and has a broad host range [1]. *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) is one of the most frequent serovars and often causes self-limiting gastroenteritis in humans. *S*. Typhimurium can also invade normally sterile sites, resulting in invasive diseases such as bacteremia and meningitis [2]. *S*. Typhimurium ingested in contaminated food or water travels down the intestinal tract to the large intestine, its primary site of replication. Intestinal inflammation is often regarded as a central host response in pathogen colonization of the intestinal tract during *S*. Typhimurium infection [1]. Therefore, discovering more detailed mechanisms by which the typical enteric pathogen interacts with the host in the gut is critical for developing novel antimicrobial strategies.

The circadian clock is present in almost all living organisms, allowing the anticipation of daily environmental changes and promoting optimal physiological function [3]. In higher organisms such as mammals, almost all aspects of physiology are regulated under circadian control, including sleep–wake cycles, blood pressure, hormonal levels, and metabolic and immune functions [4]. At the molecular level, the circadian clock machinery comprises transcriptional activators, including circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1), operating together as a heterodimer that activates the transcription of clock-controlled genes. Conversely, repressors including period (PER) and cryptochrome (CRY) work to inhibit the activity of the CLOCK/BMAL1 complex. This transcriptional-translational feedback loop system generates cyclical patterns of expression in clock-controlled genes [5]. In addition, the repression of BMAL1 by the core clock component REV-ERBα serves as an accessory feedback loop that increases the stability of circadian oscillators [6]. Previous studies have demonstrated that the circadian clock is associated with the host response to specific pathogens, including Gram-positive (e.g., *Listeria monocytogenes* and *Streptococcus pneumonia*) and Gram-negative (e.g., *S.* Typhimurium) bacteria, demonstrating the importance of the circadian rhythm in immune functions during infection [7–9]. During infection with the airborne *Streptococcus pneumonia* pathogen, targeted ablation of *Bmal1* in bronchiolar epithelial cells resulted in abnormal C-X-C motif chemokine ligand 5 (CXCL5) production and enhanced neutrophil recruitment [8]. Growing evidence indicates that the circadian clock can regulate the strength of the immune response to pathogenic challenges and the susceptibility of organisms to bacterial infection. However, the molecular mechanisms and targets underlying the differential responses to enteric infections remain poorly understood.

Nucleotide-oligomerization domain (NOD)-like receptors (NLRs) constitute a specialized group of intracellular proteins crucial for orchestrating the innate immune response of the host against infections [10]. NLRP6 belongs to the NLR family of proteins and is considered as a critical regulator of intestinal homeostasis [11]. In steady-state conditions, NLRP6 expression is abundant in intestinal epithelial cells, where it forms a complex with ASC and caspase-1, facilitating the cleavage of pro-interleukin-18 (pro-IL-18) into biologically active mature IL-18 [12,13]. The function of NLRP6 has been attributed to the maintenance of epithelial integrity and host defenses against microbial infections [11,14]. Our recent study revealed the detrimental role of NLRP6 in host iron metabolism, promoting *S*. Typhimurium growth and virulence [15]. A growing body of evidence suggests that NLRs may be involved in maintaining circadian-mediated regulation of the host immune system. However, the relationship between NLRP6 and the host circadian clock remains unclear, especially during bacterial infections.

Among the core clock components, ligand-activated nuclear receptor and transcriptional factor REV-ERBα has been previously reported to participate in immune system regulation since it directly represses specific immune genes in macrophages [16–18]. Although REV-ERBα has been gradually associated with inflammatory factor regulation, whether it plays a crucial role in infectious disease has yet to be demonstrated. In this study, intragastric inoculation of streptomycin-pretreated mice with *S*. Typhimurium was used to induce an acute colitis model [19]. We investigated the potential role of REV-ERBα in suppressing NLRP6 transcription and the subsequent regulation of *Salmonella* infection. Our initial findings demonstrate a close correlation between the host clock system and *S*. Typhimurium infection, identifying NLRP6 as a potential mediator of this association. Furthermore, we revealed the critical role of REV-ERBα in negatively regulating NLRP6 transcription by regulating *NLRP6* promoter activity *in vitro*. In addition, Rev-erbα activation in wild-type (WT) mice by SR9009 attenuates *S*. Typhimurium infection, whereas the protective effects are absent in *Nlrp6*$^{-/-}$ mice. Our data reveal that REV-ERBα represses NLRP6 and reduces the severity of *Salmonella* infection, suggesting that effective clock regulation of the immune system is essential for defending against various pathogens.

2. MATERIALS and METHODS

2.1. Mice

Six to eight weeks old male wild-type (WT) C57BL/6 and *Nlrp6* knockout mice (*Nlrp6*$^{-/-}$) characterized elsewhere, were used [15]. The C57BL/6 genetic background *Nlrp6*$^{-/-}$ mice were gifted by Professor Ying Xu (Cambridge-Suda Genome Resource Center, Soochow University, Suzhou, China). *Nlrp6*$^{-/-}$mice were generated by crossing *Nlrp6*$^{+/-}$ male mice with *Nlrp6*$^{+/-}$ female mice and the WT mice generated were used as control mice. Animals were bred and maintained under 12-h light/dark cycles in specific pathogen free conditions. All experiments were performed using co-housed littermate control mice. Animals were fasted for 4 h before intragastric administration. Approval was obtained from the Animal Experimental Committee of Soochow University for all procedures conducted (202105A0462, 202110A0319).

2.2. Bacterial strain and in vivo infection

The WT *S*. Typhimurium strain SL1344 was kindly provided by Professor Qian Yang (Nanjing Agricultural University, Nanjing,

China). When infected, SL1344 cultures grown overnight in Luria-Bertani (LB) medium were diluted to 1:100 and incubated to a logarithmic stage. After washed with sterile PBS, the amount of SL1344 in the fresh suspension was estimated and adjusted based on absorbance at 600 nm. For *in vivo* infection, mice were orally pretreated with 20 mg/mouse streptomycin for 24 h, then orally infected with 10^8 colony-forming units (CFUs) of SL1344. To assess the bacterial numbers of *S*. Typhimurium, the tissues were collected and then added to PBS containing 0.5% tergitol and 0.5% heat inactivated fetal bovine serum (FBS; Biological Industries) for homogenization. The bacterial load per gram of tissue wet weight was measured and calculated on a *Salmonella-Shigella* agar plate at an appropriate dilution. To investigate the role of Rev-erbα *in vivo*, mice were intraperitoneally administrated with 50 mg/kg SR9009 (Selleck; S8692) or vehicle once daily for 7 days prior to *Salmonella* infection, and SR9009 dosing was continued along with *Salmonella* infection. For hematoxylin-eosin (H&E) staining, Colon tissues of mice were collected and fixed in 4% paraformaldehyde overnight at room temperature. After dehydrated in ethanol, tissues were embedded in paraffin, and then cut into sections of 6 μm thickness, finally stained with H&E.

2.3. Isolation of intestinal epithelial cell

Intestinal epithelial cells were isolated from ileum or colon tissues as previously described [20]. Briefly, ileum or colon tissues were dissected and flushed with a solution of 0.154 M NaCl and 1 mM DTT to clear fecal contents. The segments were ligated and filled with PBS, then incubated at 37 °C. After 15 min, the PBS was replaced with PBS containing 1.5 mM EDTA and 0.5 mM DTT. Following 30 min of incubation at 37 °C, the contents were collected. The cells were washed twice in PBS and harvested by centrifugation at 1300 rpm for 5 min before being used for RNA or protein extraction.

2.4. RNA isolation and quantitative real-time PCR (qPCR)

Tissues or cells were lysed in TRIzol reagent and total RNA was extracted. RNA was reverse-transcribed by a RT MasterMix kit (Applied Biological Materials). Transcript levels of various genes were assessed using SYBR Green qPCR Master Mix (Bimake) on the ViiA 7 real-time PCR system (Applied Biosystems). Specific primers (Table 1) were used to amplify targeted genes. Relative expression was determined using the $2^{-\Delta\Delta CT}$ method.

2.5. Cell culture

MCF7, 293T and Caco-2 cells were kindly provided by Professor Ying Xu and Professor Weiqi He (Cambridge-Suda Genome Resource Center). Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM; HyClone) containing 10% heat-inactivated FBS

Table 1
Primer sequence for quantitative real-time PCR (qPCR).

Primer	Sequence (5′-3′)
m*Bmal1*-F	CTCCAGGAGGCAAGAAGATTC
m*Bmal1*-R	ATAGTCCAGTGGAAGGAATG
m*Clock*-F	GGTTTGATCACAGCCCAACT
m*Clock*-R	CCTCCGCTGTGTCATCTTTT
m*Per2*-F	CTGCTTGTTCCAGGCTGTGGAT
m*Per2*-R	CTTCTTGTGGATGGCGAGCATC
m*Cry1*-F	TCCCCTCCCCTTTCTCTTTA
m*Cry1*-R	TGAGTCATGATGGCGTCAAT
m*Reverbα*-F	CAGGCTTCCGTGACCTTTCTCA
m*Reverbα*-R	TAGGTTGTGCGGCTCAGGAACA
m*Nlrp6*-F	GCTGAAGGGCTCTCAAAGCA
m*Nlrp6*-R	TCGGAAAGGTCTCGGCAAAC
m*Tnfα*-F	GGTGCCTATGTCTCAGCCTCTT
m*Tnfα*-R	GCCATAGAACTGATGAGAGGGAG
m*Cxcl1*-F	TGCACCCAAACCGAAGTCAT
m*Cxcl1*-R	TTGTCAGAAGCCAGCGTTCAC
m*Il18*-F	GCCATGTCAGAAGACTCTTGCGTC
m*Il18*-R	GTACAGTGAAGTCGGCCAAAGTTGTC
m*Ifng*-F	CAGCAACAGCAAGGCGAAAAAGG
m*Ifng*-R	TTTCCGCTTCCTGAGGCTGGAT
m*Hprt*-F	CTGGTGAAAAGGACCTCTCGAAG
m*Hprt*-R	CCAGTTTCACTAATGACACAAACG
h*NLRP6*-F	ACTGTGCCATCTGAGCAGCCTC
h*NLRP6*-R	TCACTGAGCCTGTTGTGGAGGA
h*REVERBA*-F	CTGCCAGCAATGTCGCTTCAAG
h*REVERBA*-R	TGGCTGCTCAACTGGTTGTTGG
h*β-ACTIN*-F	CACCATTGGCAATGAGCGGTTC
h*β-ACTIN*-R	AGGTCTTTGCGGATGTCCACGT

m, mouse; h, human.

and 1% penicillin-streptomycin solution under conditions of 37 °C and 5% CO_2.

2.6. Plasmid, siRNA, reagents and transfection experiments

pCMV-Tag2B was kindly provided by Professor Ying Xu (Cambridge Suda Genome Resource Center). A fragment of REV-ERBα or REV-ERBβ was inserted into pCMV-Tag2B. Plasmids were transfected into cells using Lipofectamine 2000 (Thermo Fisher Scientific) for 48 h. 50 nM negative control siRNA (NC siRNA) or REV-ERBα-specific siRNA (GenePharma) were transfected into cells using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) for 48 h. Knockdown efficiency was evaluated using quantitative PCR. Poly (I:C) (tlrl-pic) and LTA (tlrl-pslta) were purchased from Invivogen and transfected into MCF7 cells or Caco-2 cells for 48 h. SR9009 (HY-16989) and GSK4112 (HY-14414) used *in vitro* were purchased from MCE.

2.7. Enzyme-linked immunosorbent assay (ELISA)

IL-18 (E-EL-M0730c) ELISA kit was purchased from Elabscience. IL-1β (SEA563Mu) and Taurine (CEV538Ge) ELISA kits were purchased from Cloud-Clone. Clone tissues of WT and *Nlrp6*$^{-/-}$ mice were isolated every 4 h from ZT0 to ZT24 (ZT = Zeitgeber time, ZT0 is light on, ZT12 is light off). ELISA for IL-18 and IL-1β was performed using the supernatant of colon tissues. ELISA for taurine was

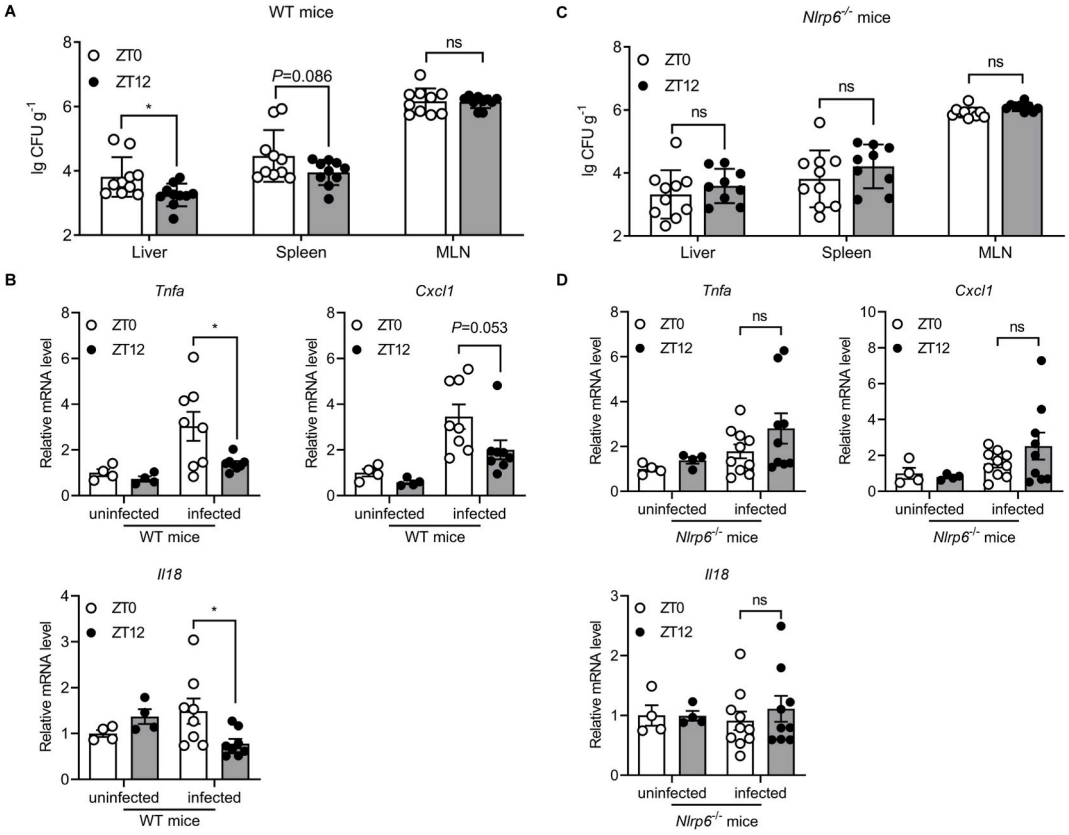

Fig. 1. NLRP6 contributes to the differential day–night response to *Salmonella* infection

(A and B) WT mice pretreated with streptomycin were orally infected with 10^8 CFU *S.* Typhimurium SL1344 at ZT0 and ZT12.

(A) The bacterial numbers of *Salmonella* in the liver, spleen and mesenteric lymph node (MLN) of WT mice were detected by plating 2 days post-infection ($n = 10$).

(B) The colonic epithelial cells of WT mice were collected, and the relative mRNA levels of *Tnfa*, *Cxcl1* and *Il18* were determined by qPCR 2 days post-infection (uninfected group, $n = 4$; infected group, $n = 8$). Results were normalized to the housekeeping gene *Hprt* and expressed as fold induction in comparison to the levels of ZT0 group of uninfected mice, which were set at a value of 1.

(C and D) *Nlrp6*$^{-/-}$ mice pretreated with streptomycin were orally infected with 10^8 CFU *S.* Typhimurium SL1344 at ZT0 and ZT12.

(C) The bacterial numbers of *Salmonella* in the liver, spleen and mesenteric lymph node (MLN) of *Nlrp6*$^{-/-}$ mice were detected by plating 2 days post-infection ($n = 9$ or 10).

(D) The colonic epithelial cells of *Nlrp6*$^{-/-}$ mice were collected, and the relative mRNA levels of *Tnfa*, *Cxcl1* and *Il18* were determined by qPCR 2 days post-infection (uninfected group, $n = 4$; infected group, $n = 9$ or 10). Results were normalized to the housekeeping gene *Hprt* and expressed as fold induction in comparison to the levels of ZT0 group of uninfected mice, which were set at a value of 1.

All error bars represent SEM. *$P < 0.05$; ns, not significant.

performed using the supernatant of colon content.

2.8. Western blotting analysis

Protein lysates were extracted using RIPA Lysis Buffer supplemented with protease and phosphatase inhibitor (Beyotime Biotechnology), and measured with a BCA assay. Cell lysates were separated on SDS-PAGE electrophoresis and transferred to PVDF membranes (Millipore). Membranes were blocked with 5% milk for 1 h, and then incubated with the corresponding primary antibodies at 4 °C overnight. Next, membranes were incubated with secondary antibodies at room temperature for 1 h, and finally visualized using an enhanced chemiluminescence luminescence reagent (Meilunbio). ImageJ software was used to calculate the intensities of the bands. Primary antibodies used are as follows: β-actin (Sigma-Aldrich; SAB1305554), FLAG (Sigma-Aldrich; F1804), NLRP6 (Sigma-Aldrich; SAB1302240), and Rev-erbα (Proteintech; 14506-1-AP).

2.9. Luciferase reporter assay

NLRP6 promoter reporter vector was constructed with pGL3-basic backbone (Promega). 500 ng of promoter reporter plasmids, 10 ng of phRL-TK renilla plasmids, together with 500 ng expression plasmids (REV-ERBα or REV-ERBβ) were transfected into cells. Promoter activity was measured using the Dual Luciferase assay kit (Promega). Firefly luciferase activity was normalized to phRL-TK renilla activity. Results were showed as fold induction compared to baseline levels (value designated as 1).

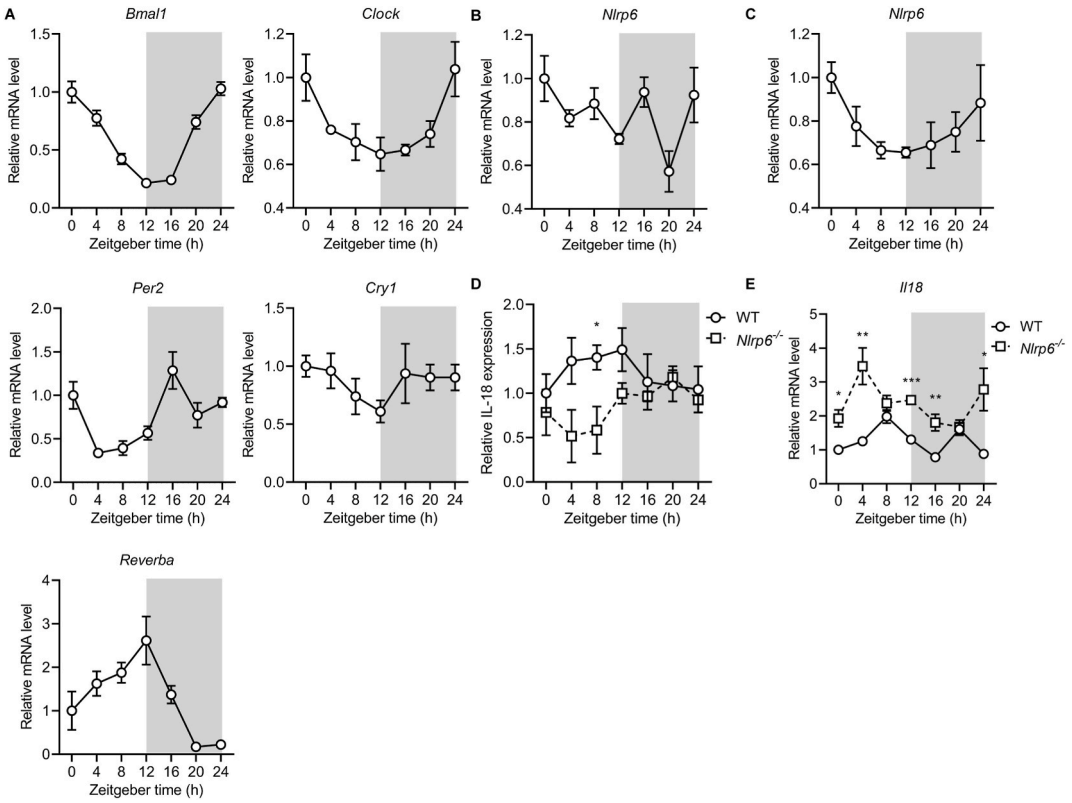

Fig. 2. Rhythmic expression of the *Nlrp6*gene in IEC

(A) The colonic epithelial cells of WT mice were collected every 4 h from ZT0 to ZT24 (ZT0, ZT4, ZT8, ZT12, ZT16, ZT20 and ZT24). The relative mRNA levels of the circadian clock genes *Bmal1*, *Clock*, *Per 2*, *Cry 1* and *Reverba* were determined by qPCR.

(B and C) The intestinal epithelial cells of WT mice were collected every 4 h from ZT0 to ZT24 (ZT0, ZT4, ZT8, ZT12, ZT16, ZT20 and ZT24). The relative mRNA levels of *Nlrp6* in colonic epithelial cells (B) and ileum epithelial cells (C) were determined by qPCR.

(D and E) The colon tissue and colonic epithelial cells of WT and *Nlrp6*$^{-/-}$ mice were collected every 4 h from ZT0 to ZT24 (ZT0, ZT4, ZT8, ZT12, ZT16, ZT20 and ZT24). (D) The relative IL-18 levels of colon tissue were measured by ELISA and normalized to the ZT0 group of WT mice, which were set at a value of 1. (E) The relative mRNA levels of *Il18* in colonic epithelial cells were determined by qPCR.

All qPCR results were normalized to the housekeeping gene *Hprt* and expressed as fold induction in comparison to the levels of ZT0 group of WT mice, which were set at a value of 1. All error bars represent SEM ($n = 4$ every time point). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

2.10. Statistical analysis

The data are expressed as mean ± SEM. Statistical analysis was performed using IBM SPSS statistics 22. Normality of numerical variables was assessed by Levene's test for equality of variances, with Mann-Whitney *U* test (non-parametric) and *t*-test (parametric) were applied for comparisons of two groups. One-way ANOVA was used to make comparisons among three or more groups. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. NLRP6 contributes to the differential day–night response to Salmonella infection

The circadian clock is known to be associated with the host response during infection with specific pathogens [21]. WT mice were infected with *S*. Typhimurium SL1344 by gavage at ZT0 and ZT12 to determine the effect of different infection times on the outcome of enteric pathogen infections. We detected colony-forming units (CFUs) in the liver, spleen, and mesenteric lymph node (MLN) of *Salmonella*-infected mice and found higher bacterial numbers in the liver and spleen of mice infected at ZT0 than in those infected at ZT12 (Fig. 1A). The inflammatory cytokine mRNA levels in colonic epithelial cells were also higher in mice infected at ZT0 than in those infected at ZT12, including tumor necrosis factor α (*Tnfa*), C-X-C motif chemokine ligand 1 (*Cxcl1*), and *Il18* (Fig. 1B). NLRP6 is an intracellular NLR highly expressed in the human and mouse intestinal tract under physiological conditions that plays a crucial role

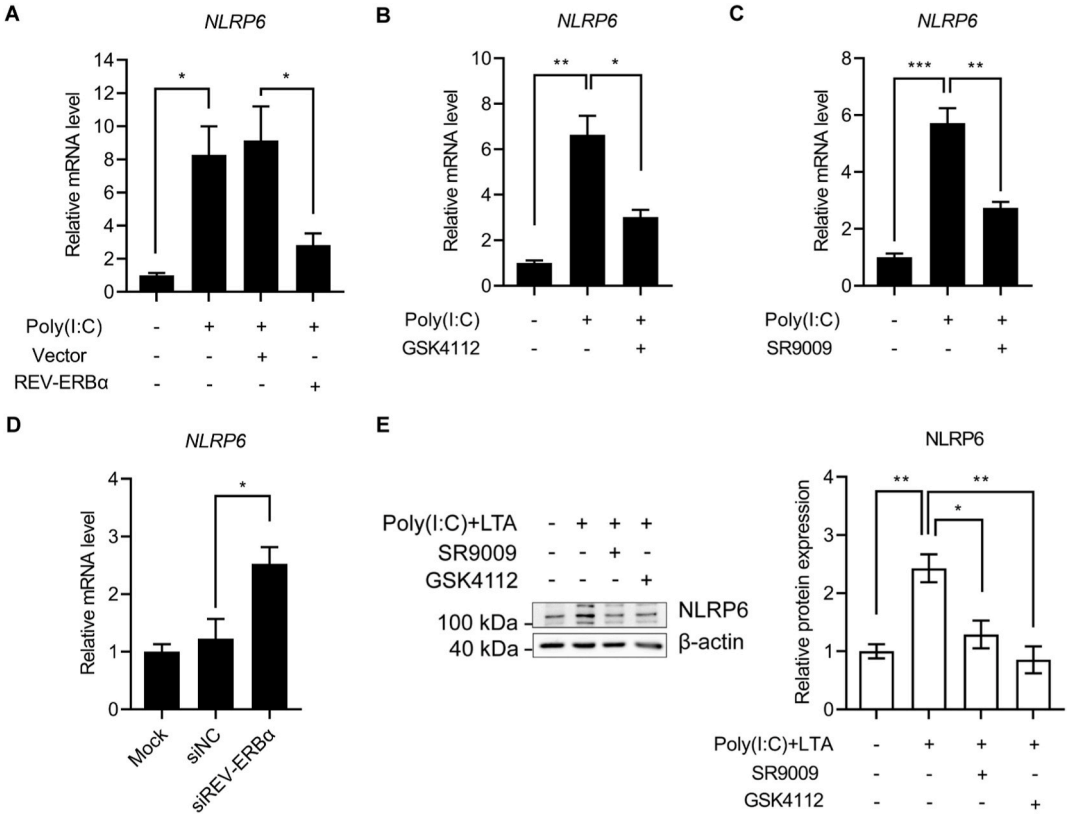

Fig. 3. REV-ERBα represses *NLRP6*expression

(A) Poly (I:C) (10 μg/mL) and the REV-ERBα expression plasmid (or vector pCMV-Tag2B, 500 ng) were co-transfected into MCF7 cells for 48 h. The relative mRNA levels of *NLRP6* were determined by qPCR ($n = 3$).

(B and C) MCF7 cells were pretreated with GSK4112 (10 μM, B) and SR9009 (10 μM, C) for 1 h, then Poly (I:C) (10 μg/mL) was transfected into MCF7 cells for 48 h. The relative mRNA levels of *NLRP6* were determined by qPCR (B, $n = 4$; C, $n = 3$).

(D) MCF7 cells were transfected with 50 nM REV-ERBα small interfering RNA (siREV-ERBα) or negative control small interfering RNA (siNC) for 48 h. The relative mRNA levels of *NLRP6* were determined by qPCR ($n = 3$).

(E) Caco-2 cells were pretreated with REV-ERBα agonists SR9009 (10 μM) and GSK4112 (10 μM) for 1 h, then Poly (I:C) (10 μg/mL) and LTA (10 μg/mL) were co-transfected into the cells for 48 h. The expression of NLRP6 protein was measured by Western blotting. One representative Western blotting image is shown ($n = 3$).

All qPCR results were normalized to the housekeeping gene *β-ACTIN* and expressed as fold induction in comparison to the levels of baseline levels, which were set at a value of 1. All error bars represent SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

in maintaining intestinal homeostasis and regulating infection. An *Nlrp6* knockout (*Nlrp6*$^{-/-}$) mouse model was generated using CRISPR/Cas9 system and infected with *S*. Typhimurium at ZT0 or ZT12 by gavage to investigate whether NLRP6 is responsible for the differential day–night response to *Salmonella* infection (figure S1A and S1B). No significant differences in the liver, spleen, or MLN bacterial numbers were observed in the *Nlrp6*$^{-/-}$ mice infected at ZT0 and ZT12 (Fig. 1C), and the cytokine expression levels in colonic epithelial cells were comparable (Fig. 1D). These data suggest that NLRP6 plays an important role in the differential day–night response to *Salmonella* infection.

3.2. Rhythmic expression of the Nlrp6 gene in IEC

The intestine plays a pivotal role in regulating the dissemination of bacteria throughout the body. Intestinal epithelial cells were isolated from WT mice every 4 h for 24 h (ZT0, ZT4, ZT8, ZT12, ZT16, ZT20, and ZT24), and circadian expression of clock genes and the *Nlrp6* gene were assessed to investigate how *Nlrp6* affects the diurnal response to *Salmonella* infection in the mouse intestinal tract. The core clock genes (e.g., *Bmal1* and *Reverba*) displayed robust diurnal expression fluctuations, and the expression of the *Nlrp6* gene showed a "W"-shaped diurnal fluctuation with an increase at ZT16 in colonic epithelial cells (Fig. 2A and B). In ileum epithelial cells, the expression of the *Nlrp6* gene displayed "U"-shaped diurnal fluctuation (Fig. 2C). Various metabolites, including taurine, carbohydrates, and long-chain fatty acids, have been identified as activators of NLRP6[22]. Interestingly, the levels of taurine in colon contents of WT mice exhibited a peak at ZT12, similar to the timing of the increased *Nlrp6* expression in colonic epithelial cells at ZT16 (figure S2A). NLRP6-dependent inflammasome activation is a key step in the maturation and release of IL-18 in intestinal epithelial cells [11–13]. Colon tissues were harvested from WT and *Nlrp6*$^{-/-}$ mice every 4 h to investigate the diurnal expression pattern of the NLRP6 downstream molecule IL-18 in the intestine. ELISA revealed rhythmic IL-18 expression in WT mice (lowest at ZT0 and highest at ZT12). In addition, the IL-18 levels in WT mice were significantly higher than those in *Nlrp6*$^{-/-}$ mice in the daytime, especially at ZT8 (Fig. 2D). Moreover, qPCR showed that the *Il18* mRNA levels in the colon epithelial cells of WT mice were significantly lower than those of *Nlrp6*$^{-/-}$ mice at multiple time points (Fig. 2E), indicating a strong correlation between the rhythmic expression of IL-18 and the dynamic levels of NLRP6.

3.3. REV-ERBα represses NLRP6 expression

REV-ERBα is a core component of the mammalian circadian clock and an important transcriptional repressor involved in regulating physiological processes, including inflammatory responses, lipid metabolism, and bile acid synthesis [23,24]. The REV-ERBα expression plasmid was constructed using the mammalian expression vector pCMV-Tag2B to explore the effects of REV-ERBα on the transcriptional levels of NLRP6 (figure S3A). According to the Human Protein Atlas website, the MCF7 cell line demonstrates relatively high NLRP6 expression levels; thus, these cells were used for the subsequent experiments. Consistent with previous reports, the qPCR results showed that the transcriptional level of *NLRP6* was activated by induction with synthetic double-stranded RNA analog Poly (I:C) [25,26]. However, the transcriptional levels of *NLRP6* were significantly inhibited upon REV-ERBα overexpression (Fig. 3A). Likewise, REV-ERBα agonists SR9009 and GSK4112 decreased the transcriptional level of *NLRP6* in MCF7 cells (Fig. 3B and C). In

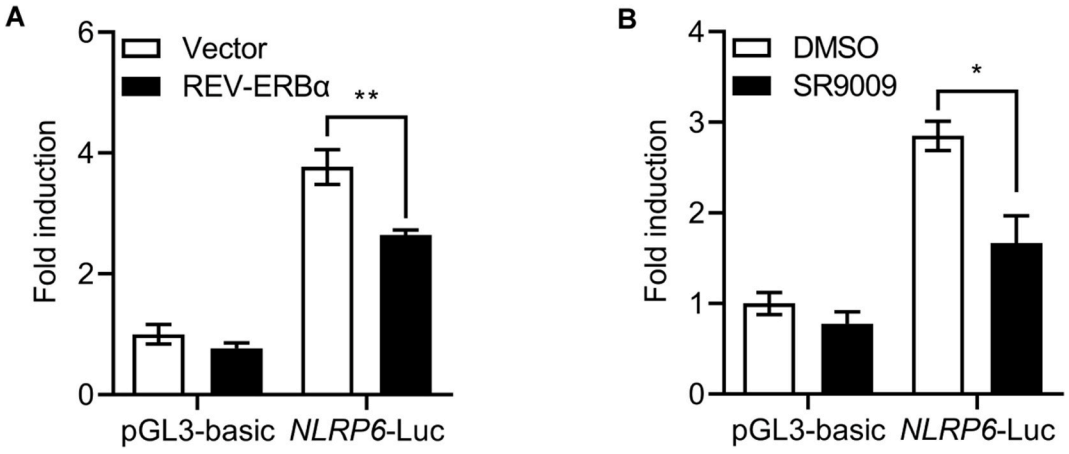

Fig. 4. REV-ERBα negatively regulates the *NLRP6*promotor
(A) 293T cells were transfected with 500 ng REV-ERBα expression plasmid (or vector pCMV-Tag2B), 500 ng *NLRP6* promoter reporter plasmid (or vector pGL3-basic) and 10 ng internal reference plasmid pRL-TK for 48 h. The activity of *NLRP6* promoter was measured by dual luciferase reporter system ($n = 3$). The vector transfection group was set to 1.
(B) 293T cells were pretreated with 10 μM SR9009 (or DMSO) for 1 h, then transfected with 500 ng *NLRP6* promoter reporter plasmid (or vector pGL3-basic) and 10 ng internal reference plasmid pRL-TK for 48 h. The activity of *NLRP6* promoter was measured by dual luciferase reporter system ($n = 3$). The vector and DMSO transfection group was set to 1.
All error bars represent SEM. *$P < 0.05$; **$P < 0.01$.

addition, the transfection of siREV-ERBα into MCF7 cells significantly increased the transcriptional level of NLRP6 (Fig. 3D and S3B). Overall, these results indicate that REV-ERBα represses *NLRP6* expression at the transcriptional level *in vitro*. The effect of REV-ERBα on the translational level of NLRP6 was further investigated in the intestinal epithelial Caco-2 cell line. Similar to the transcription data, Western blotting demonstrated that NLRP6 protein levels were significantly elevated upon co-stimulation with Poly (I:C) and LTA and decreased following treatment with SR9009 or GSK4112 (Fig. 3E), suggesting that REV-ERBα inhibits NLRP6 expression in intestinal epithelial cells. These results indicate that REV-ERBα represses NLRP6 expression.

3.4. REV-ERBα negatively regulates the NLRP6 promoter

Luciferase reporter assays were conducted to investigate the promoter activity of *NLRP6* to further explore how REV-ERBα represses *NLRP6* expression. REV-ERBα significantly inhibited *NLRP6* promoter activity (Fig. 4A), whereas REV-ERBβ (paralog of REV-ERBα) had no obvious effect (figure S4). Consistently, *NLRP6* promoter activity significantly suppressed with REV-ERBα agonist SR9009 treatment (Fig. 4B). Collectively, these results suggest that REV-ERBα directly suppresses *NLRP6* promoter activity, decreasing the NLRP6 expression level.

3.5. SR9009 alleviates the severity of Salmonella infection

The REV-ERBα agonist SR9009 has been shown to improve inflammation and the metabolic state in mice; however, its role in bacterial infection has not been reported [18,27–29]. WT and *Nlrp6*$^{-/-}$ C57BL/6 mice were intraperitoneally administrated with

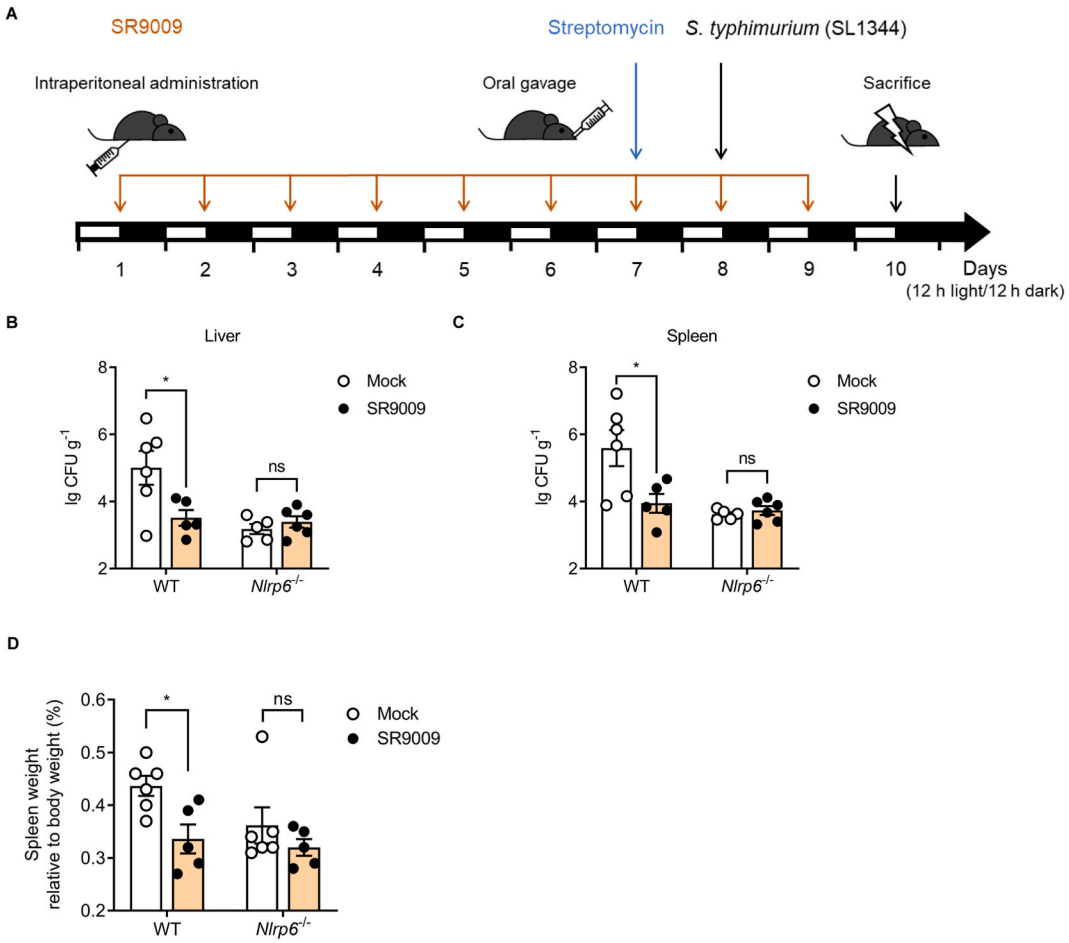

Fig. 5. SR9009 alleviates the severity of *Salmonella*infection
(A) Schematic diagram of the *in vivo* experiment. WT and *Nlrp6*$^{-/-}$ C57BL/6 mice were intraperitoneally administrated with SR9009 (50 mg/kg) daily from 1 week before until 1 day after *Salmonella* infection. Mice were sacrificed 2 days post-infection.
(B and C) The bacterial numbers of liver (B) and spleen (C) of WT and *Nlrp6*$^{-/-}$ mice were measured by plating.
(D) The ratio of spleen weight to body weight was calculated.
All error bars represent SEM ($n = 5$ or 6). *$P < 0.05$; ns, not significant.

SR9009 daily from 1 week before until 1 day after *Salmonella* infection at ZT12, when REV-ERBα protein expression levels are highest in WT mouse colonic epithelial cells, to investigate the effect of REV-ERBα on bacterial spread in *Salmonella*-infected mice (Fig. 5A and S5A). Although no significant difference was observed in bacterial numbers in the MLN (figure S5B), SR9009 treatment notably decreased liver and spleen bacterial numbers in WT mice (Fig. 5B and C). No significant differences in liver, spleen, or MLN bacterial numbers were observed in *Nlrp6*$^{-/-}$ mice with or without SR9009 treatment (Fig. 5B and C and S5B). Furthermore, the ratio of spleen weight to body weight of WT mice treated with SR9009 was significantly lower than that of mock-treated mice, while no significant differences were observed between SR9009- and mock-treated *Nlrp6*$^{-/-}$ mice (Fig. 5D). These results suggest that SR9009 intervention could reduce bacterial numbers and the degree of spleen enlargement in an NLRP6-associated manner.

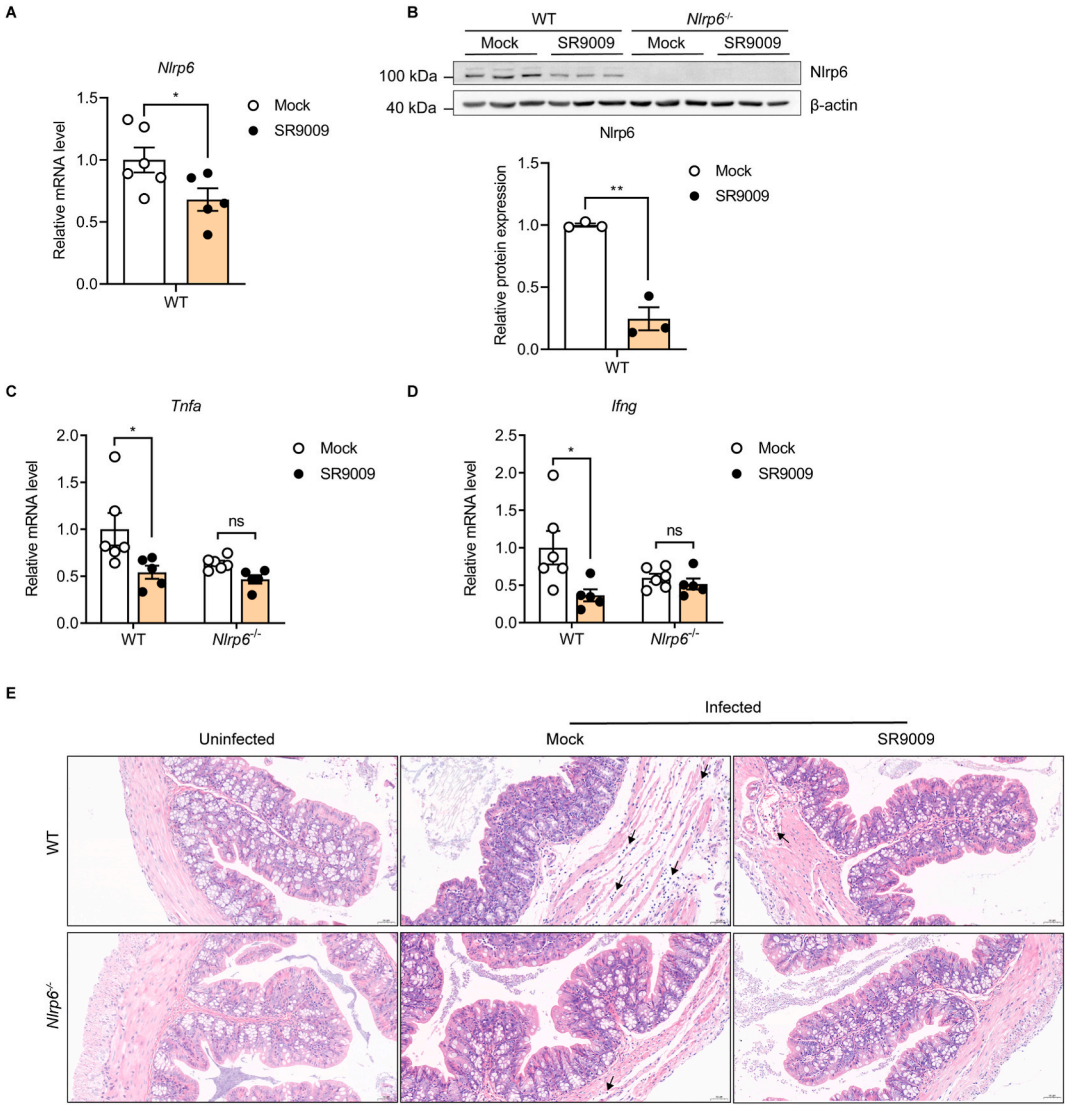

Fig. 6. SR9009 inhibits NLRP6 in IEC and regulates inflammation during *Salmonella*infection

WT and *Nlrp6*$^{-/-}$ C57BL/6 mice were intraperitoneally administrated with SR9009 (50 mg/kg) daily from 1 week before until 1 day after *Salmonella* infection. Mice were sacrificed and the colonic epithelial cells were collected 2 days post-infection.

(A) The relative mRNA levels of *Nlrp6* were determined by qPCR.

(B) The relative protein expression of Nlrp6 was measured by Western blotting ($n = 3$).

(C and D) The relative mRNA levels of *Tnfa* (C) and *Ifng* (D) were determined by qPCR.

(E) Representative hematoxylin-eosin (H&E) staining of colon sections derived from SR9009 treated or untreated WT and *Nlrp6*$^{-/-}$ mice infected with *Salmonella*; scale bar: 50 μm. Black arrows represent the infiltrated inflammatory cells.

All qPCR results were normalized to the housekeeping gene *Hprt* and expressed as fold induction in comparison to the levels of mock group of WT mice, which were set at a value of 1. All error bars represent SEM ($n = 5$ or 6). *$P < 0.05$; **$P < 0.01$; ns, not significant.

3.6. SR9009 inhibits NLRP6 in IEC and regulates inflammation during Salmonella infection

Colonic epithelial cells were obtained from our infection model to explore the correlation between the effect of REV-ERBα and intestinal NLRP6 on the bacterial spread in *Salmonella*-infected mice (Fig. 5A). qPCR and Western blotting demonstrated that the mRNA and protein levels of *Nlrp6* in WT mice treated with SR9009 were significantly lower than those in the mock group, and NLRP6 protein expression was absent from both groups of *Nlrp6*$^{-/-}$ mice (Fig. 6A and B). The *Tnfa* and interferon γ (*Ifng*) mRNA levels in intestinal epithelial cells from WT mice treated with SR9009 were significantly lower than those in cells from the mock group, while SR9009- and mock-treated *Nlrp6*$^{-/-}$ mice showed no significant differences (Fig. 6C and D). In addition, histological analysis was conducted to assess the degree of inflammation in the colon tissues of the mice. The colon of WT mice displayed an unconsolidated connective tissue structure and a greater number of infiltrating inflammatory cells, and SR9009 treatment or *Nlrp6* deficiency alleviated these infection-caused injuries (Fig. 6E). Collectively, our results suggest that REV-ERBα suppresses NLRP6 expression in intestinal epithelial cells, thereby modulating intestinal inflammation in the *Salmonella*-infected mouse model, which limits bacterial spread and mitigates colon infection.

4. Discussion

S. Typhimurium is a common pathogen in foodborne diseases and the typical Gram-negative facultative intracellular bacteria. *S*. Typhimurium infection is also a widely used model system for studying host-pathogen interactions [30,31]. In the process of the natural infection of the host by *S*. Typhimurium, the intestinal tract is not only the main place where bacteria adhere and colonize after invading the body but also an important barrier for the body to exert its antimicrobial immune function and limit infection [1,32]. Although a connection between the host circadian system and susceptibility to infection of some specific pathogens has been preliminarily established in mammals, the major effector molecules of the intestinal tract in this association have remained elusive. Here, we provide new insights into the relationship between the circadian system and infection by demonstrating that a novel NLR family member, NLRP6, is associated with the differential day–night response to *S*. Typhimurium infection. We show that the core clock component REV-ERBα negatively regulates NLRP6 transcription by regulating the promoter activity of *NLRP6*, leading to rhythmical NLRP6 expression and IL-18 secretion in the intestinal epithelial cells. This process is responsible for the differential day–night response to *S*. Typhimurium infection. Activating Rev-erbα with SR9009 in WT mice decreased the level of NLRP6 and attenuated *S*. Typhimurium infection. Our results provide new insights into the molecules linking the circadian system and intestinal infection by revealing the inhibitory effect of REV-ERBα on NLRP6 transcription and its regulatory effect on *S*. Typhimurium infection.

The mammalian circadian clock enables the synchronization of biological and behavioral activities with environmental changes, resulting in an adaptive advantage. This internal clock plays a critical role in regulating immune system function by allowing the organism to predict the timing of antigen exposure and adjust immune activity accordingly [33,34]. In this study, WT mice were orally infected with *S*. Typhimurium SL1344 at ZT0 and ZT12 to establish acute colitis models. We observed higher *Salmonella* burdens in the liver and spleen and increased expression of inflammatory cytokines in intestinal epithelial cells in the ZT0 infection group, indicating that a circadian mechanism may be involved in regulating the components of the innate immune system in response to acute *Salmonella* colitis. This finding is consistent with those presented in a previous study that also showed diurnal differences in mice in response to *Salmonella* infection [9]. However, a later report underlined the dominant role of microbiota diurnal rhythms in host immunity, which may be attributed to the different infection models [35]. Interestingly, when we performed similar experiments using *Nlrp6*$^{-/-}$ mice, no significant difference in *Salmonella* burdens or inflammatory cytokine expression levels was observed between the ZT0 and ZT12 infection groups, suggesting that NLRP6 may be an important factor in modulating differential immune responses in *Salmonella*-infected mice.

NLRP6 is expressed predominantly in mucosal tissues and is highest in the intestine; thus, the gastrointestinal tract is the most widely studied organ in NLRP6 inflammasome investigations in health and disease [12]. However, the mechanisms by which NLRP6 expression levels are regulated remain largely unclear. Previous findings suggested that NLRP6 expression is regulated at the transcriptional and posttranslational levels, which, in turn, are mainly controlled by upstream microbial and metabolic stimuli [13,36,37]. Interestingly, we observed the rhythmic expression of the *Nlrp6* gene in mouse intestinal epithelial cells. In addition, the increased expression of *Nlrp6* in the colonic epithelial cells of WT mice at ZT16 is likely attributable to the raised level of taurine in the colon contents at ZT12, since taurine is a known activator of the NLRP6 inflammasome [13,22]. IL-18 was also secreted at a high level at ZT12, which was consistent with previous studies showing that taurine promoted IL-18 production [13]. During this process, the NLRP6 inflammasome in the colon may be heavily activated and consumed, thus promoting its transcriptional level to rise at a later detection time point ZT16. Functional studies on the NLRP6 inflammasome in the intestine have elucidated its significant roles in regulating the production of IL-18 and antimicrobial peptides (AMPs) from intestinal epithelial cells, as well as in the secretion of mucus from goblet cells [38]. AMPs are thought to play a pivotal role in providing immunity to bacterial colonization on epithelia [39], but we did not observe significant difference of *Salmonella* burdens in the colon of mice between ZT0 and ZT12 infection groups (data not shown). The potential effect of AMPs on diurnal difference of infection and the underlying mechanism may be complex and need to be further confirmed in the future. In conventional *Nlrp6*-deficient mice, there is a noticeable reduction in serum IL-18 levels under steady-state conditions, along with decreased IL-18 levels observed in both the colon and serum when dextran sulfate sodium (DSS)-induced colitis and colitis-associated tumor growth are induced [13,40–42]. Consistent with these previous findings, our data further confirmed the critical role of NLRP6 in controlling epithelial IL-18 secretion. Loss of NLRP6 reduced IL-18 levels in the daytime, especially at ZT8, and altered the IL-18 secretion rhythm. Conversely, we observed the higher *Il18* transcriptional level in *Nlrp6*$^{-/-}$ mice versus WT mice at multiple time points. The possible explanation was that the *Nlrp6*$^{-/-}$ mice reactively promote IL-18

transcription, and the retarded maturation of IL-18 may further lead to its redundancy at the transcriptional level. Therefore, we demonstrated the expression rhythm of the *Nlrp6* gene in mouse intestinal epithelial cells and confirmed the *Nlrp6*-associated secretion rhythm of IL-18 in colon tissue.

A fully functioning circadian clock facilitates the mobilization of immune cells, allowing for optimal antimicrobial immune responses. In addition, the circadian clock modulates the expression rhythm of genes involved in antimicrobial responses and their products in cells, which can result in different outcomes depending on the timing of infection [43,44]. REV-ERBα, a nuclear receptor, is an important regulator in various diseases, such as metabolic disorders, inflammatory diseases, and cancers [24]. REV-ERBα directly regulates immune genes, such as *IL1b*, *TLR4*, *IL6*, *Ccl2,* and *Cx3cr1* [45–48]. Recent studies have revealed that REV-ERBα functions to suppress NLRP3 inflammasome activity, thereby mitigating the development of ulcerative colitis, peritoneal inflammation, fulminant hepatitis, and heart failure in mice [28,47,49,50]. NLRP6 and NLRP3 (highly expressed in myeloid cells) share amino-acid sequence similarities of 32% in humans and 33% in mice. The primary discrepancy lies within the domain responsible for recognizing distinct ligands [38]. Our *in vitro* study used Poly (I:C) to activate *NLRP6* gene transcription, and found REV-ERBα activation led to decreased *NLRP6* mRNA levels. In contrast, reduced REV-ERBα using siRNA effectively upregulated *NLRP6* transcription. In human colonic epithelial (Caco-2) cells, we further demonstrated the role of REV-ERBα in the negative regulation of NLRP6 protein level. Moreover, the dual luciferase reporter assay provided further evidence of the direct downregulation of *NLRP6* promoter activity by REV-ERBα. Thus, these *in vitro* data revealed that REV-ERBα modulates NLRP6 activation at the transcriptional level. NLRP6, along with NLRP1, NLRP3, NLRP7 and NLRC4, constitutes a capacity to assemble a fully functional inflammasome [12]. Whether REV-ERBα regulates the assembly of the NLRP6 inflammasome complex and the role of REV-ERBα-mediated regulation of other inflammasomes in *Salmonella* infection awaits further investigation.

Targeting REV-ERBα using SR9009 improves inflammatory and metabolic diseases in WT mice, such as DSS-induced colitis and myocardial ischemia-reperfusion [28,49]. In our *Salmonella*-infection colitis model, SR9009 was intraperitoneally administrated into mice as described in previous studies. As expected, SR9009 alleviated the severity of infection in WT mice but not in *Nlrp6*$^{-/-}$ mice. The decreased expression of NLRP6 and inflammatory factors were detected in WT mouse intestinal cells after SR9009 treatment during *Salmonella* infection. Conflicting observations in various studies indicate that NLRP6 can engage in context-specific pro- or anti-inflammatory innate immune activation [14]. Anand et al. revealed NLRP6 negatively regulates NF-κB in macrophages, but whether this negative regulatory effect is present in other cell types or tissues, such as intestinal cells, remains unclear [51]. Further studies are needed to elucidate in detail the mechanism by which SR9009-regulated NLRP6 alleviates infection severity. Investigation into the optimal dosage and timing of administration for SR9009 is essential for its potential application in antimicrobial agent development and clinical therapeutics. Several inhibitors targeting the NLRP3 inflammasome activation (e.g., MCC950 and CY-09) have been recognized and applied as promising interventions in different disease models. However, similar therapeutic interventions targeting the NLRP6 inflammasome have not been identified yet [52]. A recent study revealed that NLRP6 inflammasome complex activity regulation by Cyld-mediated deubiquitination could prevent excessive IL-18 in the intestinal mucosa, which could be exploited therapeutically [40]. Another study shed light on NLRP6 regulation by revealing that liquid–liquid phase separation (LLPS) serves as a central hub that governs NLRP6-directed host defense pathways [53]. Our findings regarding the upstream modulation of NLRP6 by REV-ERBα will help identify new regulatory checkpoints, and this mechanism is a potential therapeutic target in NLRP6-associated infectious diseases.

In summary, our findings highlight that NLRP6 serves as a crucial molecular bridge between the intestinal circadian clock and infectious colitis. Core circadian clock component REV-ERBα has a suppressive regulatory effect on NLRP6, and activation of REV-ERBα alleviates *Salmonella*-infection colitis in mice by repressing NLRP6. Targeting REV-ERBα to regulate NLRP6 is a potentially promising approach for limiting enteric infection.

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Data availability statement

Data will be made available from the corresponding authors upon reasonable request and no data was stored in any publicly available repository.

CRediT authorship contribution statement

Lanqing Sun: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Kai Huang:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Qifeng Deng:** Methodology, Investigation. **Yuan Zhu:** Investigation. **Yu Cao:** Investigation. **Kedi Dong:** Investigation. **Sidi Yang:** Investigation. **Yuanyuan Li:** Supervision. **Shuyan Wu:** Supervision. **Rui Huang:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

AMP	antimicrobial peptide
BMAL1	brain and muscle ARNT-like 1
CFU	colony-forming unit
CLOCK	circadian locomotor output cycles kaput
CRY	cryptochrome
Cxcl1	C-X-C motif chemokine ligand 1
CXCL5	C-X-C motif chemokine ligand 5
DMEM	Dulbecco's Modified Eagle's medium
DSS	dextran sulfate sodium
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
H&E	hematoxylin-eosin
Ifng	LB interferon γ Luria-Bertani
LLPS	liquid–liquid phase separation
MLN	mesenteric lymph node
NLR	nucleotide-oligomerization domain (NOD)-like receptor
PER	period
IL-18	interleukin-18
qPCR	quantitative real-time PCR
Tnfa	tumor necrosis factor α
WT	wild-type
ZT	Zeitgeber time

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