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ORIGINAL ARTICLE

Clock-controlled StAR's expression and corticosterone production contribute to the endotoxemia immune response

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Increased studies have revealed that core mammalian clock genes regulate immune functions. Previously, we reported *Per2*$^{m/m}$ mice displayed a down-regulated circadian immune response to lipopolysaccharide (LPS) challenge. However, the mediators between *Per2* and immune function and their underlying mechanisms remain unclear. In this study, serum corticosterone (CORT), a hormone which played a crucial role in immune suppression, was found to be significantly increased in *Per2*$^{m/m}$ mice compared with the one in wild-type mice following LPS administration at ZT3 and ZT8. The elevated level of serum CORT was correlated with their higher survival rate, which could be further suppressed by glucocorticoid receptor antagonist. Expression of StAR, a rate-limiting enzyme in CORT synthesis, as well as the expression of core clock genes (*Clock/Bmal1)*, was more strongly induced and longer lasting in *Per2*$^{m/m}$ mice in contrast to the ones in control mice after LPS injection. Additionally, the binding of CLOCK and BMAL1 to StAR's promoter was elevated after LPS administration, and the binding was higher in *Per2*$^{m/m}$ mice. Furthermore, loss of *Clock* function resulted in lower survival and failed to induce the serum CORT production and StAR expression in *Clock*$^{m/m}$ mice following LPS administration. Our results revealed that CORT, regulated by Bmal1/Clock transcriptional activation of StAR's expression, could function as a mediator between clock system and immune response and contribute to the endotoxemia resistance in *Per2*$^{m/m}$ mice.

Keywords: Circadian clock, corticosterone, endotoxemia, lipopolysaccharide, Per2, StAR

INTRODUCTION

The endogenous circadian clock pervasively regulates oscillations of multiple molecular and physiological processes, including heart rate, blood pressure, body temperature, hormone levels, energy metabolism and even the ability to fight against harmful invaders (Arjona et al., 2012; Logan et al., 2013; Logan & Sarkar, 2012). These daily oscillations with a period ~24 h are described as circadian rhythms (Lowrey, 2000; Reppert & Weaver, 2002), and are known to be controlled by circadian molecular machinery (two correlative feedback loops) resided in suprachiasmatic nucleus (SCN) or peripheral tissues. Heterodimeric CLOCK:BMAL1 transcriptional activator can bind E-box motifs (CACGTG) on promoters of target genes (including PERs and CRYs) and by this means drive their expressions, while the expressed PERs and CRYs can repress their own transcription by interfering with the activity of CLOCK:BMAL1 heterodimers (Lowrey, 2000; Reppert & Weaver, 2002). Thus, a self-oscillating, negatively regulated feedback loop system is established and circadian expressions of some circadian-controlled genes (CCGs) driven by this system, in turn, result in regulating rhythmic physiological processes.

Emerging lines of evidence supported the notion that one of the core clock components, *Per2*, played a crucial role in regulating the host immune functions in addition to its well-known role in the machinery of the mammalian circadian clock (Arjona & Sarkar, 2006; Boivin et al., 2003; Liu et al., 2006; Luo et al., 2009; Zheng et al., 1999). In our previous work, we found that host immune response to lipopolysaccharide (LPS), a proinflammatory endotoxin of Gram-negative bacterial which can trigger an acute toxic response in organisms, was under

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circadian control with a peak and trough of the survival rate at ZT3 and ZT8, respectively; the circadian feature in immune response was disrupted in *Per2* mutant mice. In addition, this mutant strain of mouse was more overwhelmingly resistant to LPS-induced endotoxic shock than control wild-type (WT) mice (Liu et al., 2006). The administration of LPS in mammal can activate proinflammatory cytokines (e.g. TNF-α, IL-1β, IL-6, etc.) in a short time (Alheim et al., 1997; Pekary et al., 2007; Terrazzino et al., 1997). This acute inflammation process can cause damage to infected organs, or even be lethal for infected subjects when severe inflammation occurs. This has been demonstrated in our previous study, in which the serum level of IFN-γ and IL-1β in *Per2*$^{m/m}$ mice did not increase significantly following LPS injection (Liu et al., 2006). Although these results suggested that defect in immune system in *Per2*$^{m/m}$ mice contributed to the dysfunction in LPS induced endotoxin, their underlying mechanisms remained unclear.

Corticosterone (CORT) is a predominant glucocorticoid in mice, which exhibits a daily rhythmic secretion throughout the day (Cheifetz, 1971). This hormone, primarily synthesized and released in adrenal glands, is essential for surviving potentially lethal effects of proinflammatory cytokines induced by septic shock (Paredes et al., 2007; Pekary et al., 2007). During steroidogenesis, translocation of cholesterol from cytoplasm to inner mitochondrial membrane is an essential regulatory process for CORT synthesis (Privalle et al., 1983). A key player involved in this process is steroidogenic acute regulatory protein (StAR) that is closely linked with acute induction of steroidogenesis (Clark et al., 1994; Teng et al., 2013). Clinical and experimental studies have shown that patients carrying mutation in *Star* gene and *Star*-knockout mice suffered from multiple hormonal abnormalities and suppressed levels of CORT (Caron et al., 1997; Khoury et al., 2004). Previous studies have revealed that *Star* was a clock-controlled gene and played an essential role in regulating the rhythmic production of CORT. Considering a crucial role of CORT in host immune response against acute inflammation and its circadian feature, it is suspected that this hormone, under *Star's* regulation, may contribute to the rhythmic or consistent immune response to LPS-induced endotoxic shock in WT or *Per2*$^{m/m}$ mice, and may work as a mediator between circadian clock and immune function. Under such perspective, we investigated whether CORT production, through regulation of *Star*, was responsible for arrhythmic lethal endotoxic effects of LPS in *Per2*$^{m/m}$ and *Clock*$^{m/m}$ mice.

MATERIALS AND METHODS

Animals and tissue collection

Eight to fourteen weeks old male *Per2*$^{m/m}$ (Per2^{brdm1}) (Zheng et al., 1999), *Clock*$^{m/m}$(Clock$^{\Delta 19}$) (King et al., 1997) and age- and sex-matched WT control mice, which were all C57BL/6J background, were housed under a 12 h/12 h light/dark (LD) cycle with light on at 0700 h (ZT0) or dark/dark (DD) cycle. Food and water were provided *ad libitum* and ambient temperature was maintained at 23 ± 2 °C. For WT and *Per2*$^{m/m}$ mice in DD, they were sacrificed at eight indicated time points (CT 1, 4, 7, 10, 13, 16, 19, 22) ($n = 5$ for each time point). Adrenal glands were collected and stored in −80 °C until RNA extraction. All animal studies were carried out in strict accordance with guideline for care and use of laboratory animals of the Chinese Academy of Sciences and international standards of ethical chronobiology research (Portaluppi et al., 2010).

LPS challenge and survival score

Per2$^{m/m}$, *Clock*$^{m/m}$ and age- and sex-matched WT control mice were entrained in LD for at least 2 weeks prior to LPS administration. Mice were intraperitoneally injected with a lethal dose of LPS (25 mg/kg of body weight, batch: B4:0111, Sigma, St. Louis, MO) (Liu et al., 2006). For RNA analysis, mice were sacrificed at 0 h, 0.5 h, 1 h, 3 h and 6 h after LPS challenge at both ZT3 and ZT8. For chromatin immunoprecipitation (ChIP) assay and Western blot, mice were sacrificed at 1 h after LPS or PBS (as Vehicle) administration. For each time point of each group, 3–4 mice were sacrificed. Adrenal glands were collected and stored in −80 °C freezer until RNA extraction or tissue lysing.

Five groups of LPS-injected mice including two *Per2*$^{m/m}$ groups, two *Clock*$^{m/m}$ groups and one WT group were used in our survival analysis. Of which, Mifepristone (RU486), a GR antagonist, were intraperitoneally administrated to one group of *Per2*$^{m/m}$ mice (15 mg/kg, Sigma, USA) and another group of *Clock*$^{m/m}$ mice were subcutaneously injected CORT (40 mg/kg, Tokyo Chemical Industry, Shanghai, China) prior to LPS administration. The survival rate of these five groups based on the statistics of 5–6 mice in each group was monitored in 6 h intervals after LPS injection.

Measurement of CORT

Heparinized blood samples collected at indicated time points were centrifuged at 3000 × *g* for 15 min. The concentration of serum CORT was measured in triplicate using a CORT EIA Kit (Jiang Lai Biotechnology, Shanghai, China), as in our previous study (Zhang et al., 2011). Sensitivity limit of the assay was 1 ng/ml.

Expression analysis using real-time quantitative PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). RNA quality and concentration were determined by 1% non-denaturing agarose gel and ND1000 Nano-drop spectrometer, respectively. Two microgram of total RNA was used for the first-strand cDNA synthesis using M-MLV Protoscript kit (NEB, Ipswich, MA) by following the manufacturer's protocol. Expression levels of clock genes and *Star* were determined by real time PCR using SYBR Green Mix

(Tiangen, Beijing, China) on BIO-RAD DNA Engine OPTICON 2 with dissociation curves analysis at the end of each run. PCR conditions were: 95 °C for 10 min, 40 cycles at 94 °C for 20 s, 60 °C for 60 s and signal detect for 10 s. Primers for real-time PCR were designed using ABI primer express software 2.0 (Applied Biosystems, Foster City, CA). They were: *Clock* F 5′- CTGACCGGGTT GGTTTTGAT-3′, R 5′-GGTCTTGGTGCTCATGTGCA-3′; *Bmal1* F 5′-TCGTTGCAATCGGGCG-3′, R 5′-CCGTATTT CCCCGTTCGC-3′; *Star* F 5′- GTGTGCCTTCGACCCCC -3′, R 5′-AAAGTGCTTGCTGCCTACCC-3′ and *18 S* F 5′- CTTTGGTCGCTCGCTCCTC-3′, R 5′-CTGACCGGGTTGG TTTTGAT-3′. *18 S* was used as an internal control as it was commonly held to be constantly expressed across the day. All products had validated by gel electrophoresis. Relative gene expression intensity was calculated using the Pfaffl-method (Pfaffl, 2001).

Plasmid construction

The pcDNA4-Myc-Clock and pcDNA4-Myc-Bmal1 plasmids are generous gifts from Dr. Xiaozhong Peng (Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China). The pGL3-StAR reporter vector was generated by inserting the promoter region of *Star* (−2989—0) in pGL3-basic vector. The canonical E-boxes at −2073——2068 (E2) and −2055—— −2050 (E1) upstream of the transcriptional start site in the promoter region of *Star* was mutated from CACGTG to TGAGTG by site-directed mutagenesis using the Muta-DirectTM Kit (SBS Genetech, Beijing, China) as described by the manufacturer, respectively, resulting in two mutant plasmids StAR-ΔE1 and StAR-ΔE1-ΔE2. The mutated constructs were verified by sequencing.

Cell culture and transfection

HEK293 cells in 12-well plates were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin (HyClone, Beijing, China). Cells were transfected with plasmids, containing 500 ng of reporter vectors (pGL3-StAR, pGL3-StAR-ΔE1, pGL3-StAR-ΔE1-ΔE2, respectively) and 500 ng of *Bmal1* and *Clock* expression construct, respectively, using Lipofectamine^{TM} 2000 (Invitrogen) according to the manufacturer's instructions. Cells were lysed 24 h after transfection for luciferase assay. Twenty microliters of the lysate were used for luciferase assay using a luminometer (PerkinElmer 1420 multilabel Counter Victor 3) as described by the manufacturer (Promega, Madison, WI).

Mapping of promoter binding sites and quantitative ChIP assay

For ChIP analysis, 50 mg adrenal tissue of *Per2*$^{m/m}$ and WT mice was immediately homogenized in 5 ml ice-old 1% formaldehyde, and fixed for 10 min at 37 °C. Cell membranes were lysed by adding 5 ml nuclear extracting buffer (2.2 M Sucrose, 300 mM glycine, 20 mM HEPES-NaOH, pH 7.6, 30 mM KCl, 4 mM EDTA, 1 mM DTT and 1 mM PMSF) and incubated on ice for 30 min, followed by centrifugation at 12 000 rpm for 40 min at 4 °C. The pellets were re-suspended in 1 ml SDS-sonication buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA and 1% SDS) and sonicated 15 s for 15 times, each time was spaced by 30-s intervals to the following one. Two hundred microliters of the sonicated sample were taken for phenol-chloroform extraction as input control. The rest of the sonicated chromatin fragments were diluted 10-folds with the dilution buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA and 1% Triton X-100). The chromatin solutions were pre-cleared with 40 μl salmon sperm DNA (Invitrogen) and 40 μl Protein-A Agarose beads (CALBIOCHEM, Darmstadt, Germany) for 2 h at 4 °C. After adding 14 μl anti-BMAL1 serum (produced in our laboratory), the pre-cleared solutions were incubated at 4 °C for overnight with moderate rotation. Thirty microliter salmon sperm DNA and 30 μl Protein-A Agarose beads were added and incubated for another 2 h. The protein-A-antibody-chromatin complexes were sequentially washed by low salt (50 mM Tris, pH 7.5, 10 mM EDTA, 75 mM NaCl), middle salt (50 mM Tris, pH 7.5, 10 mM EDTA, 125 mM NaCl), high-salt (50 mM Tris, pH 7.5, 10 mM EDTA, 175 mM NaCl) washing buffers and then eluted by elution buffer (50 mM NaCl, 50 mM Tris, pH 7.5, 0.1 mM PMSF, 5 mM EDTA, 1% SDS). The eluted solutions were added with NaCl and incubated at 65 °C for 4 h for reversing the crosslink. *Star* promoter was artificially divided into 11 regions (Supplementary Table 1), each was 300–500 base pairs in length. The mapping of BMAL1-CLOCK binding sites on whole *Star* promoter region was performed with these 11 pairs of primers. The 4th primer (−2333——1970) which amplified region contain the two E-boxes were used to analyze the binding of CLOCK and BMAL1 to *Star* promoter after LPS injection. Quantitative assays using real-time PCRs were performed on Opticon 2 apparatus (Bio-Rad, Hercules, CA) in triplicates.

Western blot

Adrenal glands of *Per2*$^{m/m}$ and WT mice collected at 1 h after LPS or PBS administration were lysed using Tissue Protein Extraction Kit (CWBIO, Beijing, China), boiled at 95 °C for 5 min and analyzed by 10% reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoblots were performed using anti-Clock (1:500, ab3517, Abcam, Cambridge, UK), anti-Bmal1 (1:200, ab3350, Abcam, UK) or anti-beta-actin (1:2000, #3700, Cell Signaling Technology, Danvers, MA) antibodies, respectively. Anti-rabbit IgG HRP (1:5000, #7074, Cell Signaling Technology, Danvers, MA) or anti-mouse IgG HRP (1:5000, sc-2005, Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used as secondary antibodies. The band intensity of Western blotting was semi-quantitatively analyzed using Quantity One software (Bio-Rad, USA).

Statistical analysis

Two-way analysis of variance (ANOVA) followed by Bonferroni post-test was used to compare the means of plasma CORT expression, mRNA expression of *Star* and clock genes in adrenal glands of different time points. Survival curve was analyzed with Mental-cox test. Comparison of luciferase activities was performed with one-way ANOVA. Binding activities and protein expression of Clock and Bmal1 were compared using both multiple *t*-test and two-way ANOVA. All analyses were performed using GraphPad Prism6 software (GraphPad Software, Inc., La Jolla, CA). A value of $p<0.05$ was considered statistically significant for all the studies.

RESULTS

Acute induction of serum CORT in *Per2*$^{m/m}$ mice after LPS administration

Compared with the one in WT control mice, the significantly higher level of serum CORT was found in *Per2*$^{m/m}$ mice at both time points (ZT3: $p<0.0001$; ZT8: $p<0.0001$) (Figure 1A and B), and these were correlated with their higher survival rate after LPS injection (Liu et al., 2006). And CORT level showed no significant difference between ZT3 and ZT8 in *Per2*$^{m/m}$ mice in response to LPS administration (Figure 1C). Furthermore, in WT mice, correlated with their higher survival rate at ZT3 than ZT8 (Liu et al., 2006), serum CORT concentration also showed higher level at ZT3 (Supplementary Figure 1A). This implied the role of CORT in surviving lethal effects induced by LPS from another hand. In order to confirm the role of CORT induction in the endotoxemia resistance of *Per2*$^{m/m}$ mice after LPS administration, Mifepristone, a GR antagonist, was administrated in *Per2*$^{m/m}$ before LPS injection at ZT8. It was found Mifepristone could decrease the surviving rate of *Per2*$^{m/m}$ mice even lower than the one in WT mice (*Per2*$^{m/m}$+Mife versus *Per2*$^{m/m}$: $p=0.0016$; *Per2*$^{m/m}$+Mife versus WT: $p=0.0103$) (Figure 2). These data indicated higher induction of serum CORT level in *Per2*$^{m/m}$ mice correlated with their high survival and might be essential for their surviving the potentially lethal effects of proinflammatory cytokines induced by septic shock (Paredes et al., 2007; Pekary et al., 2007).

Elevated induction of *Star*, *Bmal1* and *Clock* in *Per2*$^{m/m}$ mice followed by LPS injection

To investigate whether the difference of LPS-induced CORT secretion between WT and *Per2*$^{m/m}$ mice was associated with differential induction of *Star* expression in adrenal glands between strains, we conducted

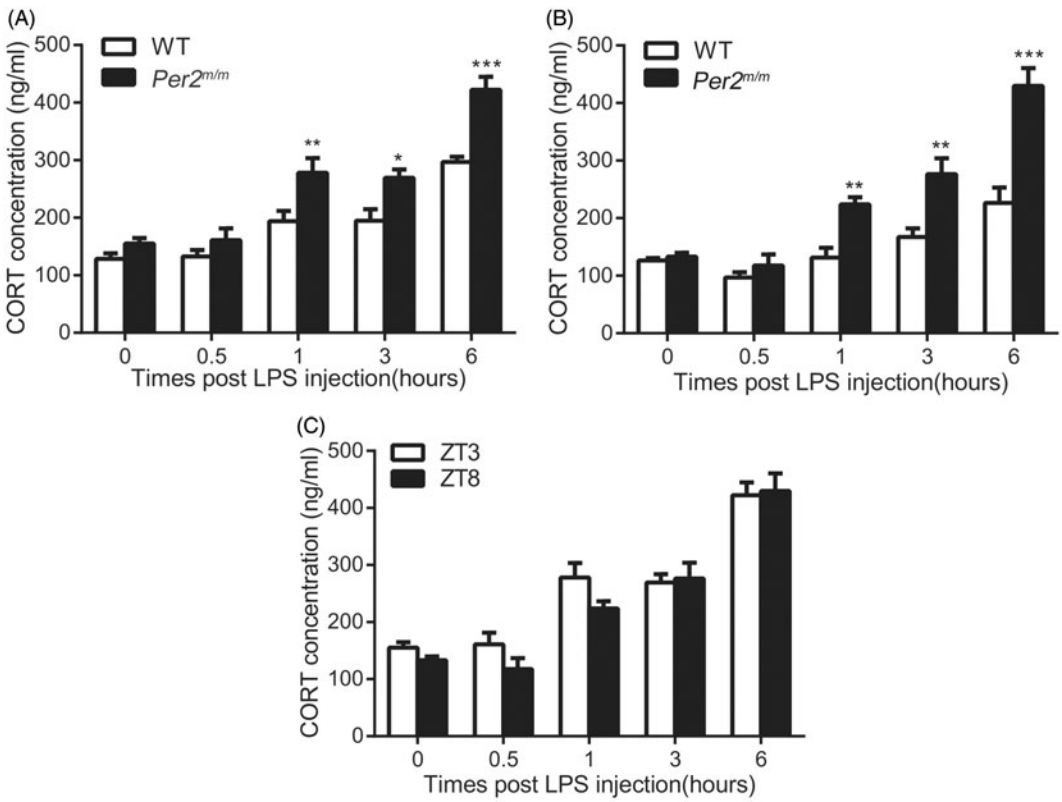

FIGURE 1. Serum corticosterone (CORT) level in *Per2*$^{m/m}$ mice compared with wild-type (WT) mice after lipopolysaccharide (LPS) injected at ZT3 (A) and ZT8 (B). CORT concentration, presented as means ± SEM ($n=4$ for each time point of each group), was measured at 0 h, 0.5 h, 1 h, 3 h and 6 h after LPS injected at ZT3 (A) and ZT8 (B) in *Per2*$^{m/m}$ and WT mice (*$p<0.05$, *Per2*$^{m/m}$ versus WT, two-way ANOVA). Mutant data from panels (A) and (B) were replotted in (C) to show a direct comparison of CORT level between ZT3 and ZT8 in *Per2*$^{m/m}$ mice (*$p<0.05$, **$p<0.01$ and ***$p<0.001$, ZT3 versus ZT8, two-way ANOVA).

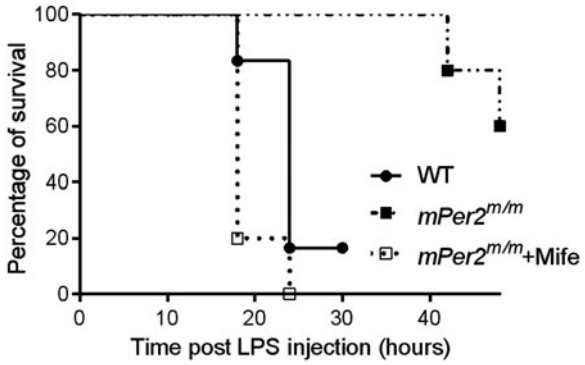

FIGURE 2. Survival rate of wild-type (WT) and *Per2*$^{m/m}$ mice injected with lipopolysaccharide (LPS). The survival rates were calculated based on six mice per group (except $n=5$ in *Per2*$^{m/m}$ group). All mice were observed at 6 h intervals for up to 48 h. *Per2*$^{m/m}$+Mife group means *Per2*$^{m/m}$ mice were given Mifepristone (RU486) (15 mg/kg) combined with the LPS solution at ZT8. (All groups have significant difference compared with WT group, $p<0.05$, Mental-cox test).

expression profiling of *Star* in adrenal glands of WT and *Per2*$^{m/m}$ mice at ZT3 and ZT8, respectively (Figure 3, Supplementary Figure 1B). As shown in Figure 3, in *Per2*$^{m/m}$ mice, *Star* expression was more highly induced by LPS treatment in contrast to that in WT mice (ZT3: $p=0.0004$; ZT8: $p<0.0001$) (Figure 3A and B), which is correlated with the higher level of acute CORT induction observed in Figure 1. LPS injection also resulted in stronger induction of mRNA expressions of *Bmal1* (ZT3: $p<0.0001$; ZT8: $p<0.0001$) (Figure 3C and D) and *Clock* (ZT3: $p<0.0001$; ZT8: $p<0.0001$) (Figure 3E and F) in *Per2*$^{m/m}$ mice in comparison with those in WT mice. In addition, in *Per2*$^{m/m}$ mice, the protein expression of CLOCK and BMAL1 presented an acute induction at 1 h after LPS injection (CLOCK, $p=0.024$; BMAl1, $p=0.011$) at ZT8; however, LPS did not induce significant changes in WT mice at the same time point (Figure 3G and H). Taken together, our results demonstrated that there was stronger acute induction in expression of *Star* as well as core clock genes (*Clock*/*Bmal1*) in *Per2*$^{m/m}$ mice after LPS treatment, and *Bmal1* and *Clock* might be implicated in the regulation of *Star*'s expression under such treatment.

The binding of CLOCK/BMAL1 to canonical E-box of *star* promoter

We then examine whether CLOCK/BMAL1 regulates *Star* gene expression. In general, rhythmic transcriptional regulation is driven by CLOCK:BMAL1 heterodimers binding to the transcriptional enhancers, such as E-box in promoter of the clock-controlled genes (Reppert & Weaver, 2002). Analysis of the promoter region of the mouse *Star* gene found 3 canonical E-boxes within the 5.3 kb region upstream of the transcriptional start site. To examine whether CLOCK:BMAL1 heterodimers drive *Star* gene transcription through these E-box enhancers, we analyzed DNA-binding activity using ChIP assay based on anti-BMAL1 antibody. *Star* promoter was divided into 11 regions (Supplementary Table 1), each was 300–500 base pairs in length. Results of ChIP experiments indicated that the highest binding activity of BMAL1-CLOCK occurred at the fourth region (−2343—1979) where harbors two E-boxes at −2073 and −2055. Due to the size of sonicated fragments, some binding activity was also found in its near region (−1954—1555), while the distal E-box at −5231 and other regions had only basal level of signals (Figure 4A).

Activation of *Star* transcription by CLOCK:BMAL1 through E-box of its promoter

A luciferase reporter system was used to validate the functional activity of E-boxes of *Star*'s promoter. Consistent with previous results (Son et al., 2008), co-expression of BMAL1 and CLOCK increased luciferase activity by 3.7-folds compared with that of control cells transfected with plasmid flanking two E-boxes in *Star* promoter region (Figure 4B). Furthermore, the CLOCK:BMAL1-dependent activation was abolished when the two E-boxes were mutated (Figure 4B). The results suggest that the canonical E box play a major role in *Star* transcription driven by CLOCK:BMAL1 heterodimers.

The binding of CLOCK and BMAL1 to promoter of *Star* was significantly induced in *Per2*$^{m/m}$ mice after LPS injection

To further clarify LPS challenge leading to *Star* increasing was through CLOCK:BMAL1 binding to its promoter region, a ChIP assay were performed using adrenal glands collected at 1 h after LPS injected at ZT8 in both *Per2*$^{m/m}$ and WT mice (Figure 4C). Our results showed that the binding of CLOCK and BMAL1 were significantly increased in both *Per2*$^{m/m}$ and WT mice after LPS injection, but the increasing were significantly higher in *Per2*$^{m/m}$ mice compared to WT mice (CLOCK: $p=0.0474$; BMAL1: $p<0.0001$) after LPS injection. These data further supported that CLOCK:BMAL1 heterodimers performed as an activator in the induction of *Star* transcription by LPS challenge.

Circadian rhythms of *Star*, *Bmal1* and *Clock* expression were disrupted in adrenal glands of *Per2*$^{m/m}$ mice

We also measured the expression of clock genes (*Bmal1*, *Clock*) and *Star* in adrenal glands of WT and *Per2*$^{m/m}$ mice under constant darkness to confirm the effect of circadian clock machinery on *Star*'s expression. As shown in Figure 5, in WT mice, circadian expression rhythms were detected for *Star* and *Bmal1* under DD cycles. In *Per2*$^{m/m}$ mice, the rhythms of *Clock* and *Bmal1* were dampened or disrupted in adrenal gland. Concurrently, the normal rhythm of *Star* was also disrupted. The results, from another perspective, confirmed the role of *Clock* and *Bmal1* in the expression of *Star* in adrenal glands.

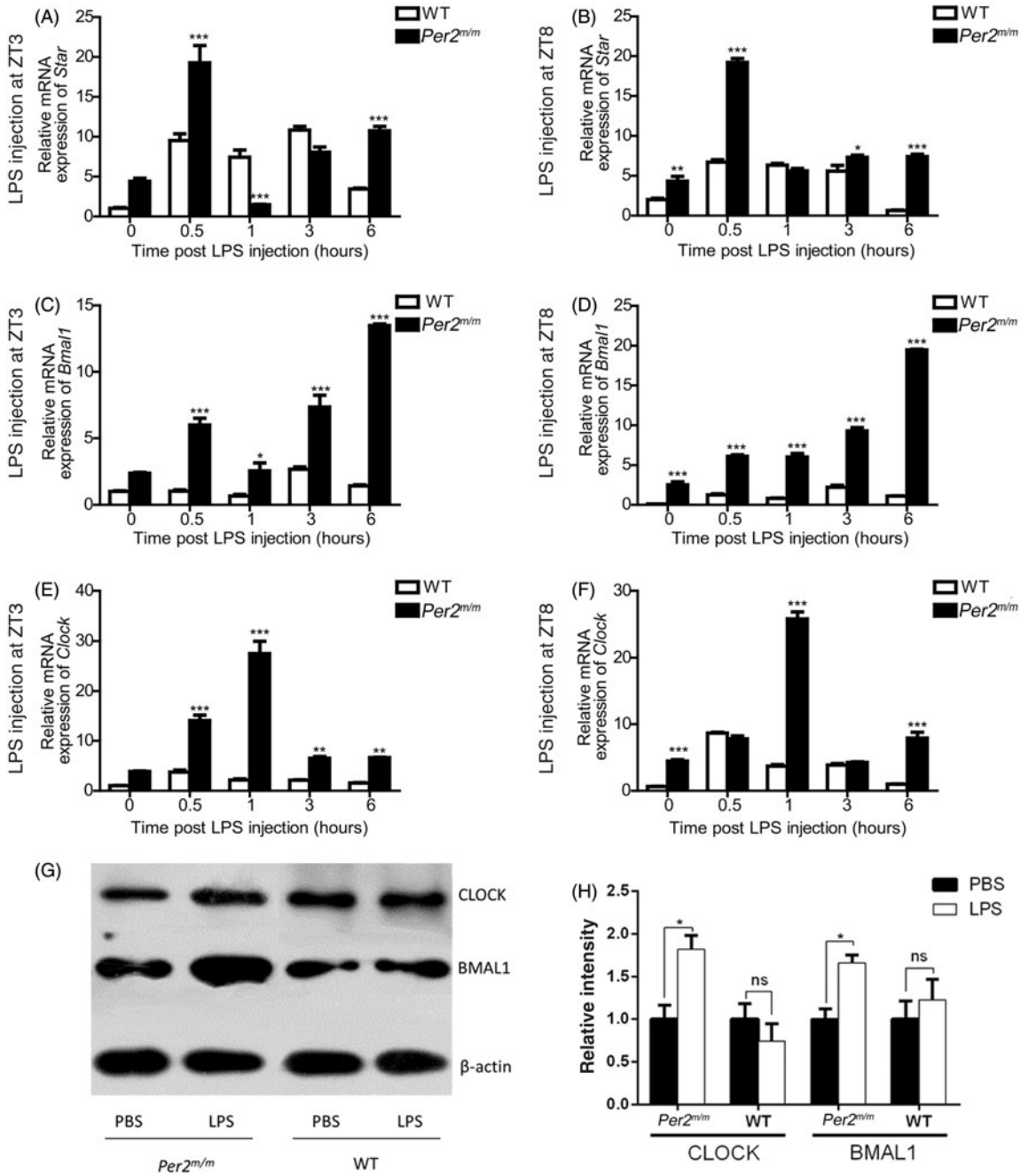

FIGURE 3. Comparison of lipopolysaccharide (LPS) induced *Star*'s, *Bmal1*'s and *Clock*'s expression in adrenal glands of *Per2*$^{m/m}$ and wild-type (WT) mice. Relative mRNA expressions of *Star* (A,B), *Bmal1* (C,D) and *Clock* (E,F), presented as mean ± SEM (*$p<0.05$, **$p<0.01$ and ***$p<0.001$, *Per2*$^{m/m}$ versus WT mice, two-way ANOVA), were measured at 0 h, 0.5 h,1 h, 3 h and 6 h after LPS injected at ZT3 (left) and ZT8 (right). (G) Protein expression of CLOCK and BMAL1 detected by Western blot. The tissues were collected at 1 h after LPS or PBS injection at ZT8. (H) Semi-quantitative analysis of the results of Western blot ($n=3$, *$p<0.05$, PBS versus LPS, multiple *t* test).

Lower induction of serum CORT production, *star* expression and survival rate in *Clock*$^{m/m}$ mice after LPS administration

To verify if CORT production and *Star*'s expression is regulated through the regulator of *Bmal1/Clock*, *Clock*$^{m/m}$ mice were used to determine the survival rate, the CORT levels, and *Star* expression in repose to LPS administration, respectively. The results showed that *Clock*$^{m/m}$ mice failed to induce the serum CORT production and *Star* expression following LPS administration (Figure 6A and C). In addition, loss of *Clock* function resulted in lower surviving rate of *Clock*$^{m/m}$ mice ($p=0.0208$), and this phenomenon was reversed by giving CORT supplementation accompanied with LPS injection (Figure 6B). All our results revealed that *Clock* was essential in modulating CORT production after LPS injection, and lower CORT induction may contribute to the lower survival in *Clock*$^{m/m}$ mice.

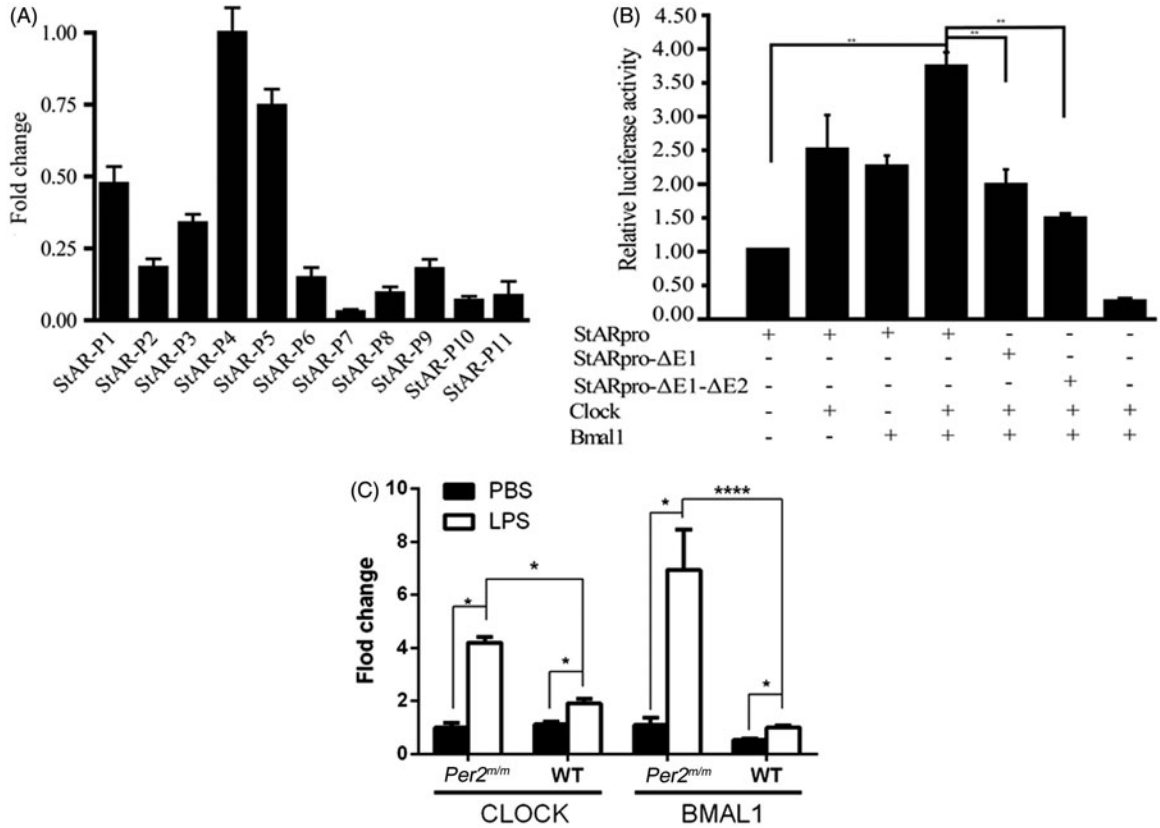

FIGURE 4. Clock:Bmal1 regulates *Star*'s transcriptional activity. (A) Binding of CLOCK:BMAL1 to canonical E-box of *Star* promoter using chromatin immunoprecipitation (ChIP) assay. *Star* promoter was artificially divided into 11 regions (Supplementary Table 1), each was 300–500 base pairs in length. (B) Activation of *Star* transcription by CLOCK:BMAL1 through E-box of its promoter. Co-expression of BMAL1 and CLOCK (but not alone) increased luciferase activity by 3.7-folds, and their activation was abolished when the canonical E-boxes was mutated (Δ). Luciferase activities were measured and signaling in each treatment was normalized to fold change of RLU compared to pGL3basic-StAR (StARpro) vector in triplicates. (**$p<0.01$, one-way ANOVA). (C) Binding of CLOCK and BMAL1 to the identified E-boxes region of *Star* promoter at 1 hour after LPS or PBS injection in adrenal gland of *Per2*$^{m/m}$ and WT mice (*$p<0.05$, PBS versus LPS, multiple *t*-test).

DISCUSSION

In our previous study, productions of serum IFN-γ and IL-1β were decreased in *Per2*$^{m/m}$ mice after the LPS injection (Liu et al., 2006). We previously hypothesized that the attenuated cytokine production was likely associated with the defective NK and NKT cell function. In another study (Luo et al., 2009), we indeed found that expression of two critical cytotoxicity receptors *Ly49c* and *Nkg2d* were lower expressed in *Per2*$^{m/m}$ mice, indicating defective NK and NKT cell function in this mutant strain. However, the mediators between *Per2* and immune function and their underlying mechanisms remained unclear. In this study, the induction of CORT in *Per2*$^{m/m}$ mice was higher than WT mice at both ZT3 and ZT8 after the LPS injection, and its induction of CORT was time-independent (Figure 1). When the CORT function was disrupted by GR antagonist, *Per2*$^{m/m}$ mice lost their strong resistant to LPS administration (Figure 2). Given by the facts of the important role of CORT in inhibition of inflammation response through suppressing the production and response of proinflammatory cytokines (Angeli et al., 1999), it is likely that the higher CORT production, combined with lower IFN-γ and IL-1β production in *Per2*$^{m/m}$, increased endotoxemia survival in *Per2*$^{m/m}$ mice.

It has been reported that adrenal peripheral clock was an important ''gating'' regulator of CORT production and controlled the autonomous circadian rhythm of CORT (Oster et al., 2006; Son et al., 2008). Circadian CORT synthesis and secretion were primarily accomplished in steroidogenic cells of the adrenal glands modulated by a master clock residing in the hypothalamic SCN through HPA axis (hormonal mechanism) (Buijs & Kalsbeek, 2001). In addition, previous work demonstrated that *Star* acted as a rate-limiting enzyme in steroidogenesis and the CLOCK:BMAL1 heterodimers regulated the autonomous circadian rhythm of CORT production through controlling *Star*'s expression (Clark et al., 1994; Son et al., 2008; Stocco, 1999). The present study also confirmed the CLOCK:BMAL1 heterodimers' binding on the two E-boxes in promoter region of *Star* (Figure 4). Additionally, we demonstrated that the

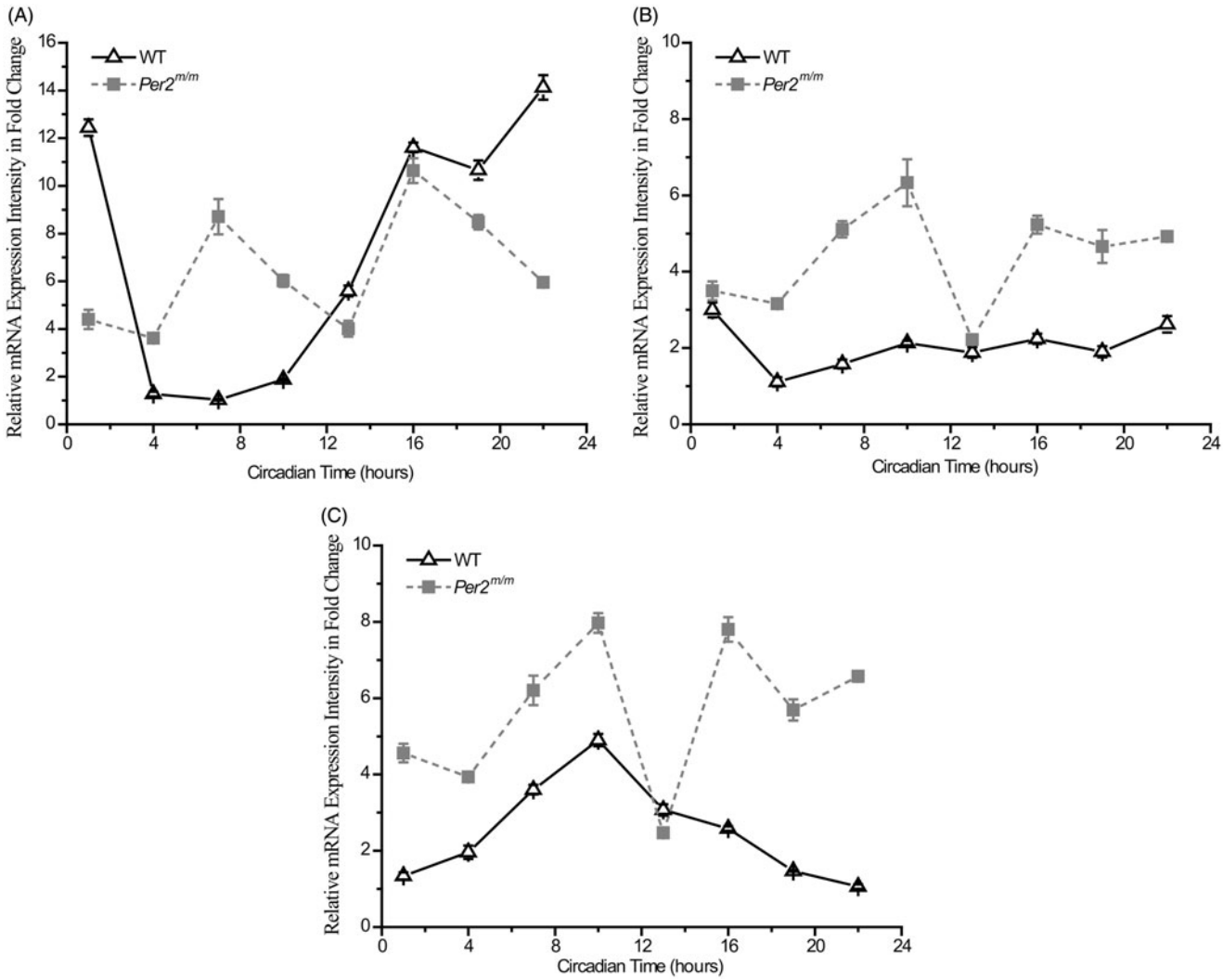

FIGURE 5. Circadian expression rhythms of *Bmal1* (A), *Clock* (B), and *Star* (C) in adrenal glands of wild-type (WT) and *Per2*$^{m/m}$ mice under constant darkness. Relative mRNA expression intensity at each time point was calculated as fold change and presented as mean ± SEM.

binding of CLOCK and BMAL1 to *Star*'s promoter was elevated after LPS administration, and the binding activity was higher in *Per2*$^{m/m}$ mice (Figure 4). Thus, we speculated that the adrenal peripheral clock may play a crucial role in modulating the timing of LPS-induced CORT production via regulating *Star* expression. For this purpose, we measured the expression profile of *Star* and clock genes (*Bmal1*, *Clock*) in both *Per2*$^{m/m}$ and WT mice adrenal glands after the LPS injection (Figure 3). Results showed, in *Per2*$^{m/m}$ mice, the mRNA expression of *Star, Clock* and *Bmal1* were more strongly induced by LPS challenge. Furthermore, the results of Western blot (Figure 4) revealed the protein level of *Clock* and *Bmal1* had similar alteration. Our studies showed loss of *Per2* function induced the higher expression of *Star*, and rendered *Bmal1* and *Clock* more susceptible to LPS, thus contributed to the increase of CORT production.

In this study, *Clock*$^{m/m}$ mice showed lower survival and basically failed to induce the production of CORT and *Star* post-LPS challenge, and supplementation of CORT accompanied with LPS administration could increase the survival rate of *Clock*$^{m/m}$ mice, these results reinforced the positive regulation of *Clock* in CORT production. Another study also discovered *Bmal1* knockout mice shown great reduction of survival to Listeria monocytogenes infection (Nguyen et al., 2013), which further support the notion that positive regulator in circadian machinery play positive role in immune response. However, it should be noted that CORT may not be the only mediator between *Clock* and immune function. This could be supported by the facts that *Clock* could directly inhibit the glucocorticoid receptor (GR) by acetylation (Nader et al., 2009). Furthermore, *Bmal1*/*Clock* heterodimer can also regulate other immune-related factors, like toll-like receptor 9 (TLR9) (Silver et al., 2012) and inflammatory monocyte chemokine ligand (CCL2) (Nguyen et al., 2013) in different tissues, to contribute to decreased septic shock under LPS challenge.

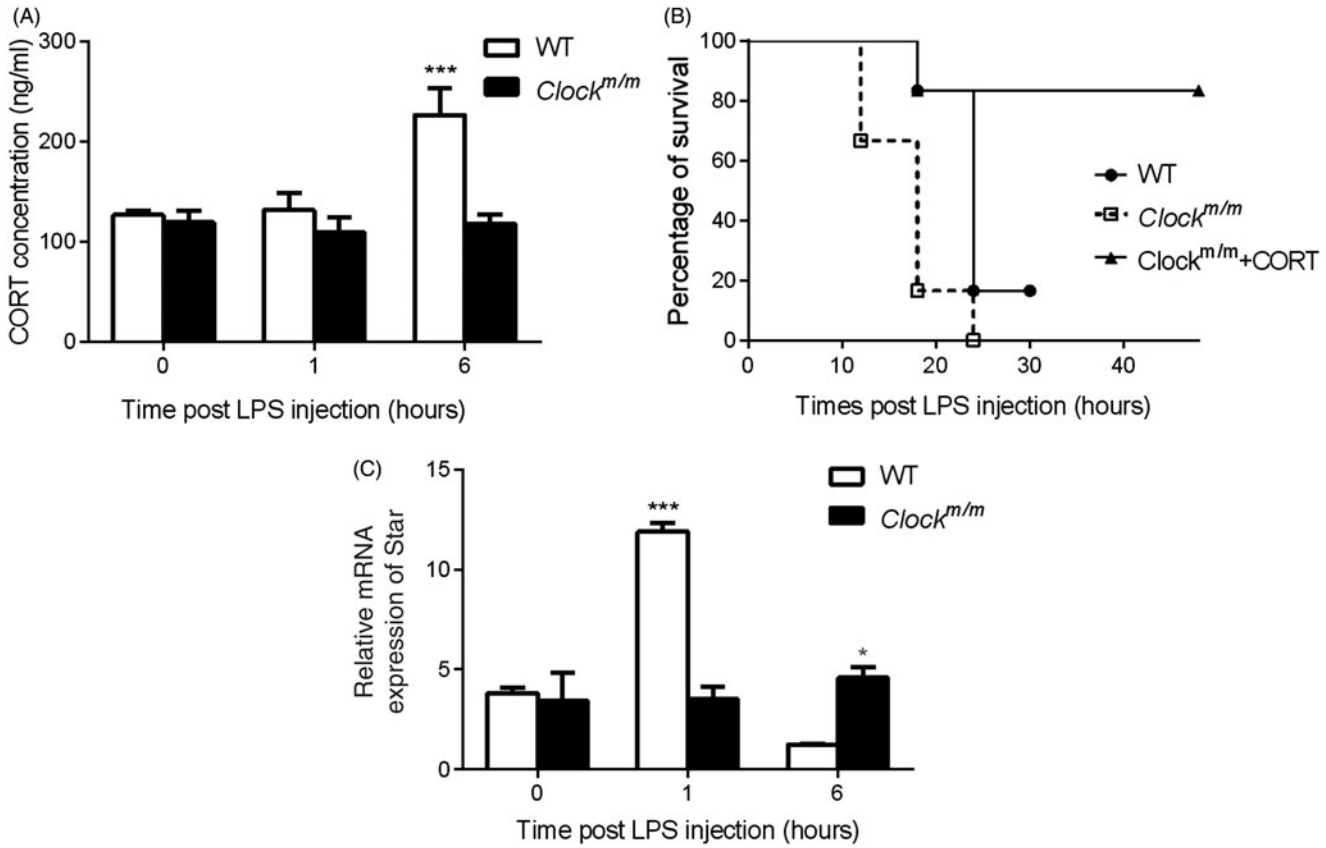

FIGURE 6. Serum corticosterone (CORT) level (A), survival rate (B) and *Star*'s expression (C) in *Clock*$^{m/m}$ mice after LPS administration. (A) CORT concentration, which presented as mean ± SEM ($n = 4$ for each time point in each group, $^{***}p < 0.001$, *Clock*$^{m/m}$ versus WT mice, two-way ANOVA), was measured at 0 h, 1 h and 6 h after LPS injection at ZT8. (B) Survival rates were calculated using three groups of mice. Six *Clock*$^{m/m}$ or WT mice were given a LPS injection only, or *Clock*$^{m/m}$ mice ($n = 6$) were injected with LPS and CORT ($p < 0.05$ *Clock*$^{m/m}$ versus WT mice and *Clock*$^{m/m}$ versus *Clock*$^{m/m}$ + CORT, Mentalcox test) (C) Relative expression of *Star* in adrenal glands of *Clock*$^{m/m}$ and WT mice after LPS challenge ($^{*}p < 0.05$ and $^{***}p < 0.001$, *Clock*$^{m/m}$ versus WT, two-way ANOVA).

In addition, recent studies revealed that *Per2* could directly interact with nuclear receptors, including peroxisome proliferator-activated receptor-alpha (PPAR-α) and REV-ERBα (Schmutz et al., 2010); the former exhibits anti-inflammatory and immunomodulatory activity (Kidani & Bensinger, 2012), and the later could modulate proinflammatory cytokines (Gibbs et al., 2012). Additionally, loss of *Per2* may also affect the expression of cryptochromes (Shearman et al., 2000), which had been reported in direct participation in glucocorticoid regulation (Lamia et al., 2011). These suggested that *Per2* might affect immune function through diversified biochemical and biological pathways in an independent manner to *Bmal1*/*Clock*.

In conclusion, our results revealed that *Per2,* through negatively regulating *Bmal1*/*Clock* transcriptional activation of *Star*'s expression, played an important role in modulating induction of CORT production, which contribute to the endotoxemia resistance in *Per2*$^{m/m}$ mice. Although explicit function of *Per2* in immune system remains to be further explored, our works could provide new insights for future investigation.

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DECLARATION OF INTEREST

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Supplementary information available online

Supplementary Table 1 and Figure 1