UNIVERSITY OF CALIFORNIA

SANTA CRUZ

BACTERIAL INFECTION RESETS THE CIRCADIAN CLOCK OF MOUSE LUNG FIBROBLASTS VIA INDUCTION OF CLOCK PROTEIN PER2

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by

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Abstract

Bacterial infection resets the circadian clock of mouse lung fibroblasts via induction of clock protein PER2

By Ti (Dung Ngoc) Lam

Circadian rhythms regulate changes in physiology and allow organisms to respond to predictable environmental demands over 24 hours. Recent studies have shown that the circadian system is an essential regulator of immune function. Additionally, disruption of the circadian clock affects our immune response. However, how bacterial infection affects our circadian clock, which affects our immune response, remains unclear.

Here, we found that the addition of heat-killed S. pneumoniae (HK Spn) to mouse lung fibroblast cells impacts their circadian rhythms through acute induction of clock protein PER2. Due to the acute induction of PER2, it resets the cells to a different phase. Moreover, HK Spn can affect the PER2 rhythm throughout the clock, and interestingly, HK Spn resets the clock of these mouse lung fibroblasts to the same circadian time. Furthermore, we also identify the dose-dependent effect of HK Spn on PER2 rhythm. Gram-positive bacteria and gram-negative bacteria are evolutionarily different from each other. Nonetheless, we find that all tested bacteria trigger the same PER2 response. Therefore, this response is an evolutionarily conserved response within all bacteria. Furthermore, we identify that TLR2 and TLR4 are not responsible for this bacterially induced PER2 induction. My work also focuses on the downstream of the signaling pathway, where we characterize that the PER2 response is post-

transcriptional and likely mTOR-independent. Together, our results advance understanding of the bidirectionality effect between circadian rhythms and bacterial infections.

Dedication

To my parents and twin sister, I couldn’t have done this without you.

Acknowledgements

I'd like to thank my mentors and colleagues. To start, I would like to thank Dr. Jacqueline Kimmey, for the opportunity to be in the lab, to do research and the mentorship and guidance to help me learn to become an independent scientist. I would especially thank the people who have contributed to this project, Dr. Carrie Partch and Priya Crosby who were not just our collaborators on this project but also great mentors. Priya especially, the biggest contributor to this project. I would not be able to learn these techniques and data analysis without her time, patience, and guidance. Jacqueline, Carrie and Priya have mentored me since the beginning, and I would not be able to move the project forward without any of them. I would like to thank Dr. Douglas Kellogg for being on my thesis committee and QE committee, his input and feedback. I would also like to thank my lab mates from both Kimmey and Partch lab. I have learned so much from each of them. I am thankful for their emotional and intellectual support.

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lab mate, my lab sister. We have gone through so much together, doing rotation together, joining the lab together. Stephanie and Dori, thank you for always supporting and cheering for me!

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Chapter 1: Introduction

1.1 Circadian clock

All living organisms go through environmental rhythms such as a daily transition from light to dark and changing temperature. From cyanobacteria to humans, most organisms have developed an internal timing system, known as the circadian clock, that allows them to anticipate changes and align internal biological processes with these environmental rhythms. Circadian rhythms are 24-hour cycles of physical, mental, and behavioral changes that regulate homeostasis and changes in physiology. Past studies have shown that how disruption of circadian rhythms affects sleep (Hirano et al., 2016; Patke et al., 2017), metabolism (Guan et al., 2018), Alzheimer's disease (Holth et al., 2019), aging (Zwighaft et al., 2015), cancer (Kettner et al., 2016; Kiessling et al., 2017), innate immunity (Gibbs et al., 2011), and susceptibility to infections (Edgar et al., 2016; Kiessling et al., 2017). Therefore, the circadian system plays an essential role in regulating physiology.

A circadian rhythm is defined with three key characteristics: (1) has a period of approximately 24 hours without any external cues, that can (2) be entrained by external cues, and is (3) temperature-compensated to maintain the same period over a broad range of physiological temperature (Pittendrigh, 1960). Furthermore, the effects of an external cue on circadian rhythms can be studied by identifying the impacts on the features of a periodic wave such as period, phase shift, and amplitude (Figure 1). The period (τ) is the amount of time it takes for a cycle to return to the equivalent point on the oscillation. As an example, the period can be defined by the time it takes from peak

to peak or trough to trough. Phase shift (θ) is a change (either in advance or delay) in the organism's point in the oscillation in response to the environment cue. A representation of phase shifting can be plotted as phase response curve (PRC), which shows the phase shift in hours vs. the time of stimulus addition in circadian time (CT); circadian time is a quantification of time which is defined by the organism's endogenous timing, without reference to any external cues. The amplitude (A) is the distance from peak to trough.

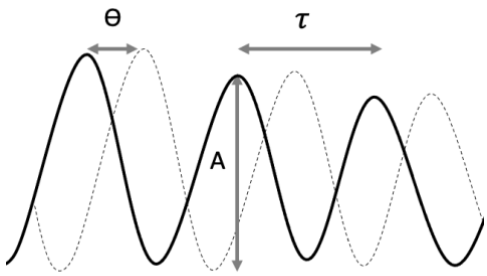


Figure 1. Features of periodic waves. τ denotes period. θ denotes phase shift. A denotes amplitude.

1.2 Molecular clock mechanism

In mammals, the central clock resides in the suprachiasmatic nucleus (SCN) in the hypothalamus, ensuring synchronization with the light and dark cycle. The retina receives the light and relays the signal to the SCN, which adjusts the circadian phase then communicates this information to other clock sets in the peripheral organs via humoral pathways (Yamazaki et al., 2000; Yoo et al., 2004). At the molecular level, the circadian clock consists of multiple transcription factors that result in autoregulatory transcription-translation feedback loops (TTFLs) (Takashashi et al.,

2017). Figure 2 shows the overview of the TTFLs of mammalian circadian clock. Positive regulators are the brain and muscle arnt-like protein 1 (BMAL1) and circadian locomotor output cycles kaput (CLOCK). They bind to E-boxes in the promoter of circadian clock genes, including *period* (*Per*), *cryptochrome* (*Cry*), and nuclear receptors (*Rev-Erb*), to induce their expression. PER and CRY are negative regulators of CLOCK:BMAL1; upon translation, PER and CRY form a complex with casein kinase 1 (CK1), translocate to the nucleus, and interact with CLOCK:BMAL1 to inhibit its own transcription. When PER and CRY are degraded by the proteasome, this lifts the inhibition; as a result, CLOCK:BMAL1 activates a new round of gene expression. This cycle takes about 24 hours. In the second loop, retinoic acid-related orphan receptors α, β, and γ (RORs) are driven by D site-binding protein (DBP) and Nfil3, and RORs bind to the ROR-response elements in the BMAL1 promoter, thus inducing its expression. On the other hand, REV-ERB, whose gene expression is driven by BMAL1-CLOCK, can inhibit ROR activity on BMAL1 expression and CLOCK expression (Crumbley and Burris, 2011).

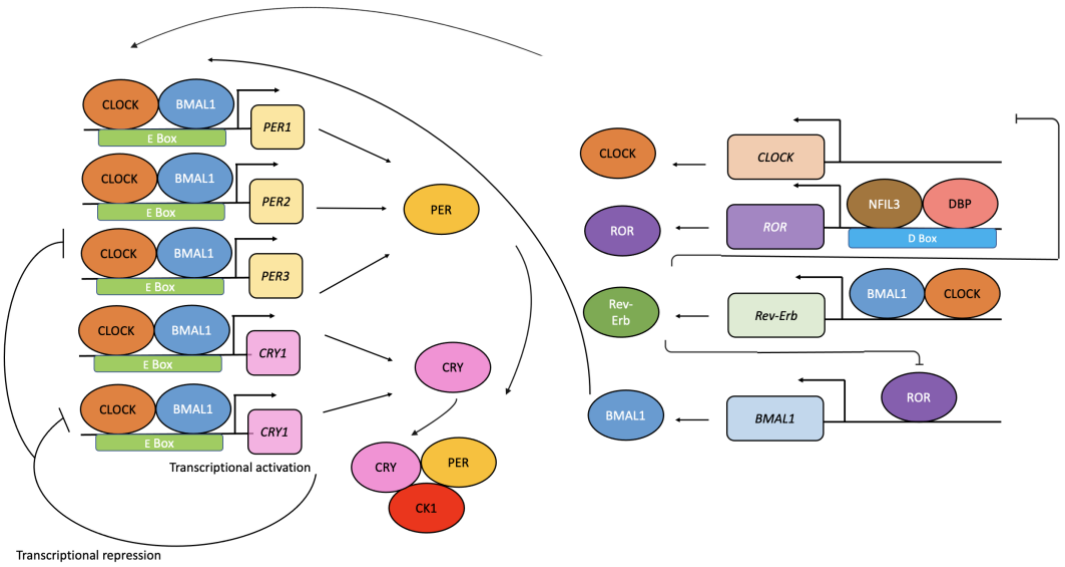


Figure 2 Transcriptional and translational feedback loops (TTFLs) of the mammalian circadian clock. The first loop includes BMAL1, CLOCK, PER, CRY. The second loop includes BMAL1, CLOCK, Rev-Erb, and RORs.

1.3 PER2::LUC fibroblasts and temperature entrainment

The Period genes family includes *Per1*, *Per2*, and *Per3*. Past studies have shown that *Per1* and *Per2* play an essential role in maintaining circadian rhythmicity, whereas *Per3* is not crucial compared to the other 2 Per genes in mice (Albrecht et al., 2001; Bae et al., 2001). This study on the effects of bacterial addition on circadian rhythms mainly focuses on PER2 expression. To investigate these effects of bacterial exposure in vitro, lung fibroblasts from PERIOD2::LUCIFERASE (PER2::LUC) mice were used. The PER2::LUC mice were generated to have the promoter and codons for Per2 fused to the luciferase gene's codons. Upon translation, the resulting protein is a fusion protein of PER2 and luciferase, and this fusion protein allows us to measure

PER2 expression by monitoring luciferase. Hence, PER2::LUC cells provide information about the quantity of PER2, which cannot be obtained from a Per2-Luc transcriptional reporter with the coding region of luciferase fused only to the promoter of Per2. The cells were extracted from lung tissues from PER2::LUC mice, and cells underwent serial passage to become immortalized.

Circadian rhythmicity in a cell-autonomous fashion can be synchronized by temperature cycles (Brown et al., 2002). Since all mammals have temperature fluctuation in a circadian manner, Brown and colleagues demonstrated that these temperature cycles could help maintain the rhythmicity of circadian oscillators in peripheral tissues and cells. Therefore, the PER2::LUC fibroblasts in this study were cultured at 37ºC for 12 hours and 32ºC for 12 hours to ensure these cells were synchronized before any experimentation. The peak of PER2::LUC expression of the lung fibroblasts in this study is denoted as CT12. Hence, in this study, CT is used as normalized hours relating to CT12 as the peak of PER2::LUC expression.

1.4 Regulation of PER2

Several past studies have been focused on the transcriptional regulation of PER2. It has been shown that the activators CLOCK and BMAL1 interact with chromatin and chromatin-modifying complexes such as the histone acetyltransferases p300 and CREB-binding protein, respectively (Etchgaray et al., 2002; Curtis et al., 2004; Lee et al., 2008; Hosoda et al., 2009). As an example, light induces *Per2* in the SCN (Albrecht et al., 1997). In addition, the glucocorticoid hormone analog dexamethasone induces the expression of circadian genes, including *Per2*, in cultured

rat-1 fibroblasts and shifts the phase of circadian gene expression in peripheral tissues (Balsolobre et al., 2000a,2000b). Furthermore, it has been shown that glucocorticoid-induced Per2 induction is mediated by the BMAL1-dependent binding of a glucocorticoid to the E-box in the 5' upstream region of Per2 (Cheon et al., 2013). On the other hand, glucose can reset cellular circadian oscillation by downregulating the level of *Per2* (Hirota et al., 2002).

There are also several post-transcriptional mechanisms that regulate Per2 transcripts. One example is that mRNA stability of Per2 is modulated by polypyrimidine tract-binding protein, which binds to the 3' untranslated region (3'UTR) of Per2 (Woo et al.,2009). Furthermore, it has been shown that miRNA24 binds to the Per2 3′-UTR, which downregulates PER2 expression (Yoo et al., 2017). Moreover, Crosby and colleagues found that insulin and insulin-like growth factor (IGF1) reset the circadian clock *in vitro* and *in vivo* by upregulating the translation of existing Per2 transcripts via PI3K activation and mTOR activation, and downregulating of PER-cognate miRNA levels (Crosby et al., 2019). In addition, light can directly increase PER2 translation (Cao et al., 2015). Following translation, PER2 is regulated by a CK1-dependent phosphoswitch, which can either stabilize or degrade PER2 depending on the site that it is phosphorylated (Zhou et al., 2015). All in all, these transcriptional, post-transcriptional, and translational and post translational mechanisms all play a role in underscoring the tight control of PER2 abundance as a key factor in the clock.

1.5 Interplay between circadian clock and bacterial infection

In recent years, it has been discovered that there is a link between the biological clock and many aspects of our immunity. For example, several groups have previously reported variations in disease severity for infections occurring at differing times throughout 24 hours. Gibbs and colleagues demonstrated the release of inflammatory mediators and antibacterial responses to Streptococcus pneumoniae (*S. pneumoniae* or Spn) is mediated by a circadian clock within epithelial cells (Gibbs et al., 2014). Additionally, disruption of the circadian clock affects the immune response; specifically, deletion of a core clock gene, Bmal1, in macrophages results in enhanced antibacterial immunity (Kitchen et al.,2020). Nonetheless, how bacterial infections affect the circadian clock remains unknown. Therefore, the object of this study is to elucidate how heat-killed bacteria affect the PER2 expression in mouse lung fibroblasts.

Chapter 2: Materials and Methods

2.1 Cell culture

PER2::LUC mouse lungs were gifted from David Welsh's lab (UCSD). Fibroblasts were isolated and underwent serial passage to become immortalized, and these steps were done by Priya Crosby from the Partch lab (CB3). The extraction of cells from lung tissues was done following this instruction (Seluanov et al., 2010), with a modification: primary fibroblasts were generated at atmospheric O_2 with 5% CO_2 instead of 3% O_2 and 5% CO_2. After cells became immortalized, they were cultured in a 175 cm^2 flask (Thermo Scientific) in 30 ml DMEM (GIBCO) supplemented with 10% Hyclone TM fetalclone TM III (GE Healthcare, Lot# 16777-240) and 1% penicillin-streptomycin at 37°C-32°C temperature cycling, 5% CO2. Cells were split when at about 90% confluence. Cells were seeded and grown in temperature cycles of 12 hours at 37°C and 12 hours at 32°C until they are 100% confluent.

2.1.1 Cell culture – experimental

Cells were grown to confluence in 35 mm dishes (about $1x10^6$ cells) in DMEM (GIBCO) supplemented with 10% Hyclone III serum (GE Healthcare, Lot# 16777-240) and 1% penicillin/streptomycin. Cells were cultured in temperature entrainment cycles of 12 hours at 37°C followed by 12 hours at 32°C for a minimum of 72 hours. Once confluent, cells were then changed to serum-free "Air Medium" (Hastings et al. 2005) containing either a B27 (Gibco, 17504044) or NS21 (made in-house) supplement. Dishes of cells were sealed with vacuum grease and circular 40 mm coverslips (Fisher

Scientific). Cells were then transferred to a Lumicycle-32 (Acimetrics) or an ALLIGATOR (Cairn Research) luminometer to record the bioluminescent level before stimulation.

2.1.2. "Air medium"

Bioluminescence experiments were performed using "Air medium" (Hastings et al.2005). 1000 ml of "Air Medium" stock includes:

DMEM (powder, D2902)	12 g
100 g/L glucose	40 ml
1M MOPS	20 ml
Penicillin/ Streptomycin	10 ml
7.5% $NaHCO_3$	4.7 ml
Millipore water	up to 1000 ml

The stock was adjusted to pH 7.6 with 10 M NaOH.

2.1.3. Serum replacement NS21

It was made following Chen et al. 2008. All the components are from Sigma except holo transferrin from Calbiochem, Cat#. 616414

		Final concentration	
	Cat. #	µg/ml	µM
Albumin, bovine	A4919	2500	37
Catalase	C40	2.5	0.01
Glutathione (reduced)	G6013	1	3.2
Insulin	I1882	4	0.6
Superoxidase dismutase	S5395	2.5	0.077
Holo transferrin	616424	5	0.062
T3 (triiodol-l-thyronine)	T6397	0.002	0.0026
L-Carnitine	C7518	2	12
Ethanolamine	E9508	1	16
D(+)-galactose	G0625	15	83
Putrescine	P5780	16.1	183
Sodium Selenite	S9133	0.01435	0.083
Corticosterone	C2505	0.02	0.058
Linoleic acid	L1012	1	3.5
Linolenic acid	L2376	1	3.5
Lipoic acid (thioctic acid)	T1395	0.047	0.2
Progesterone	P8783	0.0063	0.02
Retinol acetate	R7882	0.1	0.2
Retinol, all trans (vit. A)	95144	0.1	0.3
D,L-alpha-Tocopherol (vit. E)	95240	1	2.3
D,L-alpha-Tocopherol acetate	T3001	1	2.1

2.1.4 Preparation of "Air medium" working stock

Before recording, 50 ml of working stock was made, consisting of:

Air medium stock	46.5 ml
Glucose (27.8 mM)	2 ml
Firefly luciferin (1 mM)	500 µl
B27/NS21 (Life Technologies/in house)	1 ml

The working stock was adjusted with 5 M NaCl to bring the osmolarity up to 350 mOsm/L and then 0.22 µm sterile filtered before use.

2.2 Culture and heat killing *Streptococcus pneumoniae*

Streptococcus pneumoniae D39V was cultured in Todd-Hewitt Broth containing 2% yeast extract, called THY medium. Working stock of the pneumococcal cells was prepared by collecting cells at OD600 0.4 and then suspended with fresh THY medium with 18% glycerol and stored at -80°C. *Streptococcus pneumoniae* were cultured until OD600 0.4 and heat-killed in a large batch for multiple experiments. Then, bacteria cells were pelleted after centrifugation. The pellet was then resuspended in PBS at 1/100 of the starting THY volume. 20 µl of the resuspended cells were collected prior to heat killing to enumerate bacterial cells via serial dilution and plating on Columbia Blood Agar plates (5% sheep blood). After incubation overnight at 37°C with 10% CO2, plates were counted to determine colony forming units (CFU). The remaining resuspended cells were boiled at 100°C for 30 minutes. Heat-killed bacteria were plated on blood agar plates to confirm no viable bacteria remained.

2.2.1. Addition of heat-killed bacteria or a bacterial component

About 48-72 hours post media change, PER2::LUC cells were exposed to either PBS or heat-killed bacteria or a bacterial component. The temperature was maintained during the treatment of the circadian experiment by placing dishes of cells on an isothermal pad. Prior to using, the pad was heated to 39°C using a microwave. The solution inside the pouch, which is in solid form at room temperature, became a clear fluid. The pad was then let cool to 37°C. Then, the pad was ready to use. After the bacterial addition, cells were returned in either Lumicycle or ALLIGATOR to record the response.

2.2.2 CpG DNA extraction

A bacterial culture D39V of 400 ml THY was grown at 37°C overnight. When OD600 was 0.4, bacterial cells were harvested and resuspended in protoplast buffer (20% sucrose, 20 mM Tris-HCL, 10 mM MgCl2, 10 mM MgCl2, and DI water). Mutanolysin (0.1%) was added, and the culture was incubated for 60 minutes at 37°C. Proteinase K (0.1%) was added and incubated for 45 minutes at 55°C. Bacterial cells were harvested, and DNA was extracted using Qiagen Miniprep kit (#27106) and provided protocol. DNA was measured using a NanoDrop and concentrated using a Speed Vacuum.

2.2.3 Bacterial components (CpG DNA, LTA, LPS)

	Supplier	Cat#
Lipoteichoic acid (*B. subtilis*)	Sigma	L3265
Lipopolysaccharide (*E. coli,* strain 0111: B4-TLR4*)*	Invivogen	Tlr1-3pelps

Lipopolysaccharide was given from Susan Carpenter's lab (MCDB).

2.2.4 Addition of pharmacological agents or inhibitors

When cells were treated with an inhibitor, cells were exposed to the inhibitor 30 minutes before bacterial addition. This was done on an isothermal pad to maintain cells at 37ºC. Cells were then returned to Lumicycle or ALLIGATOR. Cells were then treated with heat-killed bacteria on an isothermal pad at 30 minutes after adding the inhibitor. Then, cells were returned in either Lumicycle or ALLIGATOR to record the response.

	Supplier	Cat#
α-amanitin	Santa Cruz	SC-202440
cycloheximide	Sigma	C7698
Leptomycin B	Cayman	87081-35-4

2.3. qPCR for mRNAs

Cells were harvested in 300 µl lysis buffer from Zymo Research Quick RNA miniprep kit (Thermo Fisher). RNA extraction was done using the protocol provided from the same RNA miniprep kit. On-column DNA digestion was carried out.

cDNA synthesis was performed using an iScript cDNA synthesis kit (Biorad). A total 20 µl reaction included 4 µl 5x reaction mix, 1 µl reverse transcriptase, 200 ng RNA, nuclease-free water to make up the amount. This step was done on ice. Then, samples were incubated in a thermal cycler using provided protocol from the kit: 5 minutes at 25ºC, 20 minutes at 46ºC, and 1 minute at 95ºC.

qPCR was done using Quantabio PerfeCTa® SYBR® Green SuperMix Reaction Mixes. Each sample well was 12.5 µl, which consisted of 6.5 ul SYBR Green Supermix, 1.0 µl primer mix (forward and reverse), 2.5 µl of nuclease-free water, and 2.5 µl of cDNA. The plates were sealed and centrifuged briefly and placed in the Biorad qPCR machine. The reaction set was 95°C for 3 minutes, 40 cycles of 95°C for 15 seconds and 58°C for 45 seconds, and 72°C for 30 seconds. A melt curve was then determined by heating to 95°C for 15 seconds, 55°C for 15 seconds before returning to 95°C for a further 15 seconds. Data were analyzed using CFX Manager software, using the determined threshold. All quantities were normalized to RNS18 quantity.

Sequences and annealing temperatures for all primers used were as follows (Crosby et al., 2019):

Primer: Per2 Forward: CCTACAGCATGGAGCAGGTTGA

Primer: Per2 Reverse: TTCCCAGAAACCAGGGACACA - 58°C

Primer: Rns18 Forward: CGCCGCTAGAGGTGAAATTC

Primer: Rns18 Forward: TTGGCAA ATGCTTTCGCTC-58°C

2.4 Data analysis

2.4.1 Circadian data analysis

Bioluminescence traces of cells obtained from Lumicycle, or ALLIGATOR were fitted with damped cosine waves using the following equation:

$$y = mx + c + Amplitude \cdot e^{-kx} \cdot \cos\left(\frac{2\pi(x - phase)}{period}\right)$$

Y is the signal. X is the corresponding time. Amplitude is the height of the peak of the waveform above the trend line, k is the decay constant (such that 1/k is the half-life), phase is the shift relative to a cosine wave, and the period is the amount of time taken for a complete cycle to occur (Crosby et al., 2019). This enables an estimation of the period, amplitude, and phase to be made, with goodness-of-fit determined using R^2 values (Crosby et al., 2019). PER2 expression was measured by using the area under curve analysis feature from Prism. To measure the acute PER2 expression following bacterial addition, the area of interest was defined at the time of treatment to 4 hours post-treatment. This enables an estimation of PER2 expression. Luciferase activity or

bioluminescent signal is presented as relative light units (RLU). The area under the curve is given as arbitrary units (AU).

2.4.2 Statistics

Raw traces are presented as mean ± SEM unless otherwise stated, and statistical significance of mean differences determined using student's t-test or ANOVA as appropriate. Statistical analyses were performed using GraphPad Prism Version 9.2.0. P values are reported using the following symbolic representation: ns = $p>0.05$, * = $p\leq0.05$,** = $p\leq0.01$, *** = $p\leq0.001$, **** = $p\leq0.0001$.

Chapter 3: Results

3.1 Heat-killed *Streptococcus pneumoniae* (HK Spn) affects circadian clock of mouse lung fibroblasts

The addition of HK Spn to the culture medium affects circadian rhythms of temperature-synchronized mouse lung fibroblasts as determined by acute induction of clock protein PER2. As the bioluminescence traces show in Figure 3A, upon bacterial addition, there is an increase in PER2 expression (shown in blue). This acute induction of PER2 was quantified by the area under the curve analysis for samples collected at 4 hours post bacteria addition (Figure 3B). The area of interest was defined at the time of treatment to 4 hours post-treatment since the peak of acute induction is about 4 hours. Moreover, HK Spn increases the amplitude in the bacterial-treated cells (Figure 3C). Although HK Spn enhances PER2 expression, it does not affect the period (Figure 3D).

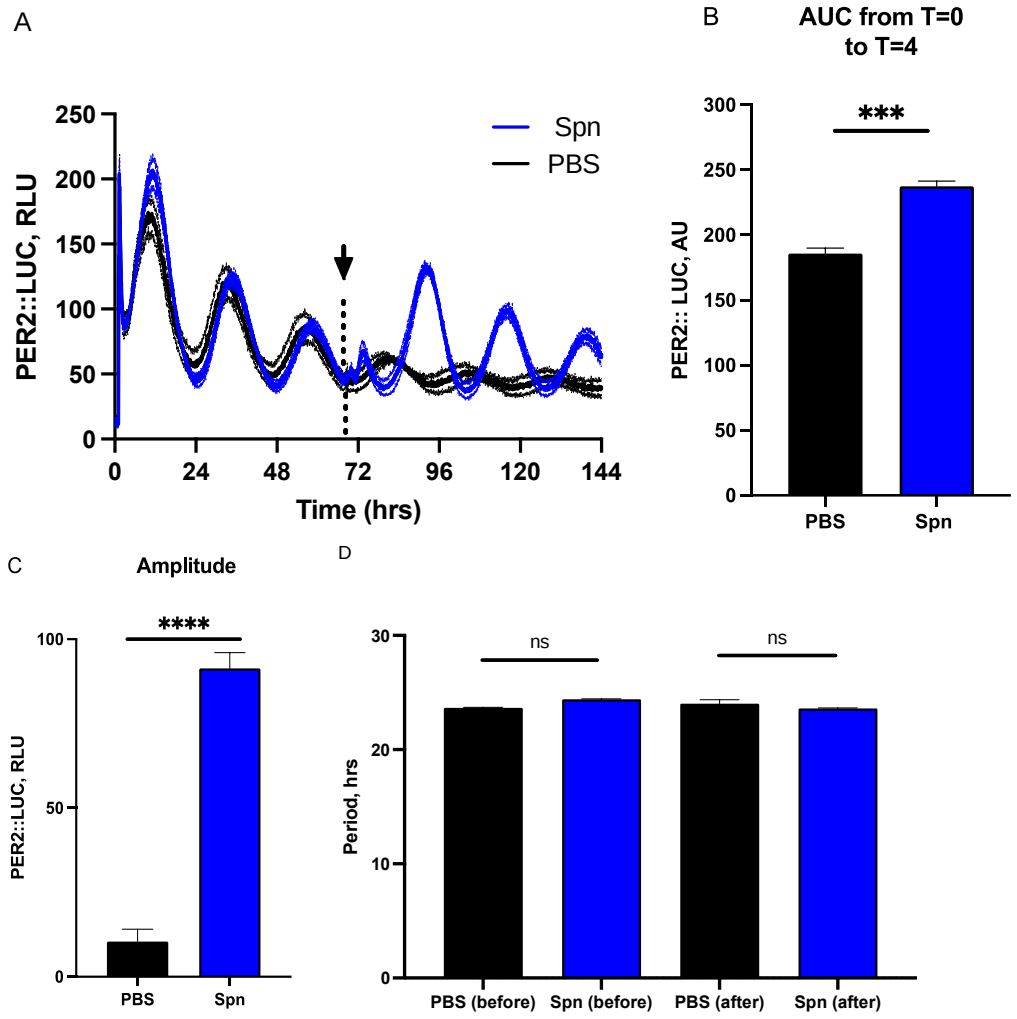


Figure 3. Spn induces PER2 expression in temperature synchronized mouse PER2::LUC lung fibroblasts. A). Trace of PBS treated fibroblasts is shown in black, and the trace of HK Spn treated fibroblasts is shown in blue. The dotted line denotes when PBS or Spn ($0.75x10^8$ CFU/ml) is added. This is representative data. Technical replicates n≥3. Traces are shown as mean ± SEM. B). Quantification of PER2 expression as area under the curve analysis at 4-hour post infection. C). Quantification of amplitude. D). Quantification of period before and after bacterial exposure.

The effect of HK Spn on phase was studied at different infection times throughout the circadian cycle: 12 pm (Circadian Time CT22), 4 pm (CT2), 8 pm (CT6), 12 am (CT10), 4 am (CT14), and 8 am (CT18); each infection was done on a different set of cells culture dishes. Thus, HK Spn was added to cells at different phases,

as shown in Figure 4A-F. These data indicate that although the acute induction of PER2 is smaller or bigger, which depends on the time of the infection, HK Spn can affect the expression of PER2 in these mouse lung fibroblasts throughout the clock. Nevertheless, the increase in amplitude is not always observed. For example, there is no enhanced amplitude when cells were exposed at CT18 (Figure 4F). Therefore, the effect of HK Spn on PER2 amplitude depends on the time of infection.

The PRC shows the changes in phase versus the time of bacterial exposure. The phase-shifting is either advanced (shifting forward) or delayed (shifting backward) at different infection times. The PRC also presents the time window for no phase shifting, which is known as the "dead-zone". Interestingly, the phase response curve (PRC) (Figure 4G) shows that regardless of the time of bacterial addition, HK Spn resets the clock of these mouse lung fibroblasts to the same circadian phase, which is CT10. For example, when cells were exposed to HK Spn at CT6, the delta phase was 4 hours in advance. Thus, the resulting phase is CT10. These cells will have the same resulting phase as those cells which were treated at CT10. Moreover, when cells exposed to HK Spn at CT10, there is no phase shifting occurs. Further experiments are needed to study the biological meaning of why cells shift phase to CT10 upon bacterial exposure.

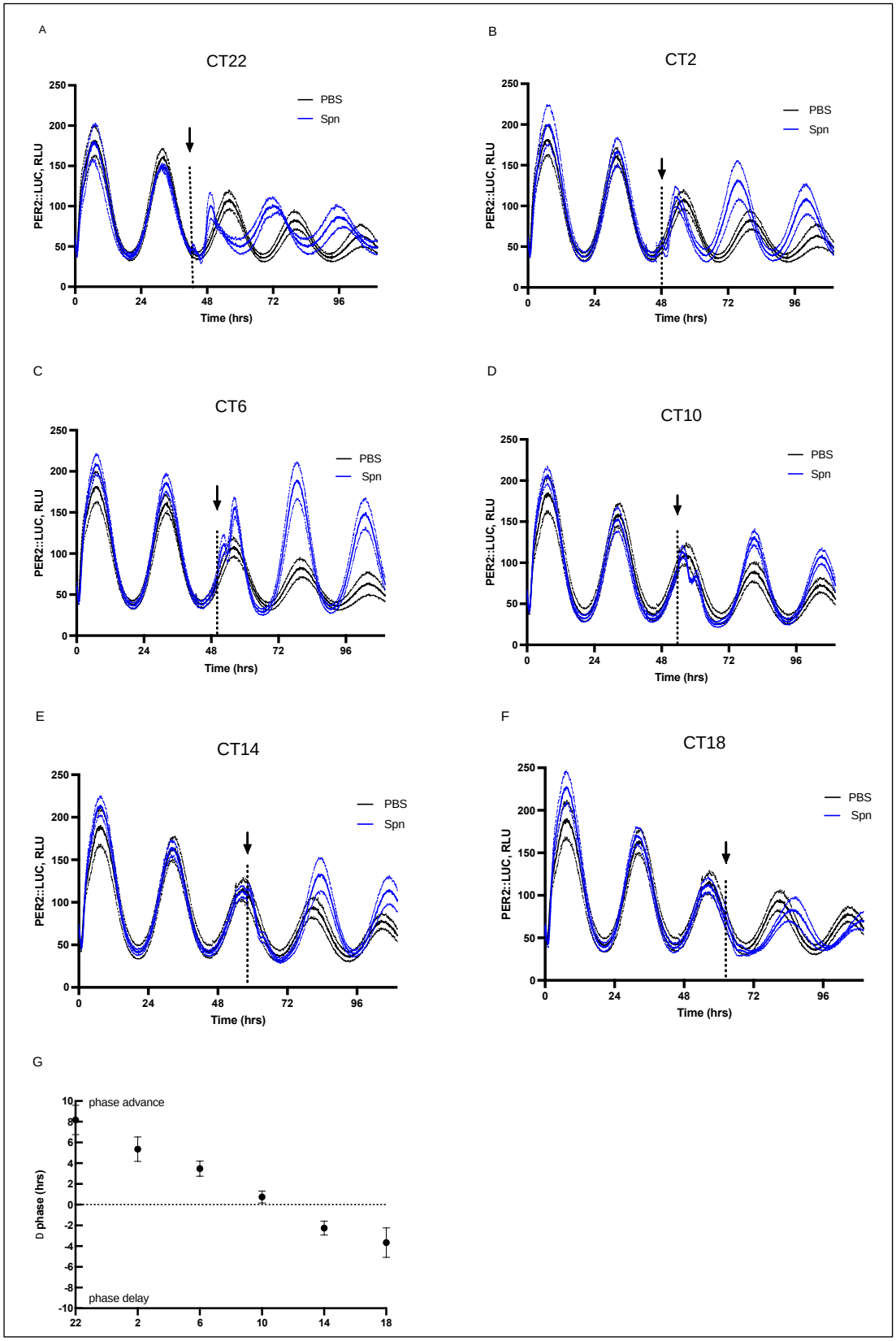

A
CT22
PBS
Spn
PER2::LUC, RLU
Time (hrs)
B
CT2
C
CT6
D
CT10
E
CT14
F
CT18
G
phase advance
phase delay
D phase (hrs)

Figure 4. The addition of HK Spn to different sets of temperature synchronized mouse PER2::LUC lung fibroblasts at different times throughout the 24-hour cycle. A). CT22, B). CT2, C). CT6, D). CT10, E). CT14, F). CT18. Trace of PBS treated fibroblasts is shown in black, and the trace of HK Spn treated fibroblasts is shown in blue. The dotted line denotes when PBS or HK Spn ($0.75x10^8$ CFU/ml) is added. Technical replicates n≥3. Traces are shown as mean ± SEM. G). Phase response curve (PRC) of bacterial addition presents the phase shifts in hours (y) vs the time of bacterial addition (x). Positive Δ phase (hours) denotes phase advance (shifting forward). Negative Δ phase (hours) denotes phase delays (shifting backward). Technical replicates n≥3)

Next, I wanted to know whether PER2 response is similar in lung fibroblasts that were derived from different PER2::LUC mice. Thus, I used PER2::LUC fibroblasts from 4 different mice, namely mice 2,3 and 4 (Figures 5B-D). Figure 5A shows traces of the lung fibroblasts from mouse 1, which are the cells used in Figures 3 and 4. There is an acute induction of PER2 observed in mouse 1. Figure 5 shows that PER2 responses upon bacterial addition in these different cell lines are varied. As shown in Figure 5B, the PER2 induction is negligible in mouse 2 cells compared to the one in mouse 1 (Figure 5A). In addition, there is no clear induction peak in fibroblasts from mice 3 and 4 (Figure 5C and 5D). There is a limitation in this experiment: mouse 1 cells were not entrained as long as they were used in the other experiments (Figure 3 and 4) as this cell line (mouse 1 cells) was used for multiple experiments at once. They might not be 100% confluent when I did this experiment. Thus, this could be why the PER2 induction in mouse 1 cells (Figure 5A) was minimal compared to the ones we see from Figure 3 and 4 experiments. Nevertheless, the differences in PER2 response that we see from different cell lines were not due to the confluency of mice 2,3,4 cells because they were 100% confluent and cultured for a longer time.

In addition, regarding the phase, the shifting in mouse 1 (Figure 5A) is not apparent, as shown in Figure 3. This was probably a combined effect of these cells were not being entrained long enough and the exposure itself. On the other hand, the phase-shifting observed from mouse 3 fibroblasts is minimal yet without an induction. As D'Alessandro and colleagues pointed out that a change in PER2 is sufficient to shift the phase of these cells (D'Alessandro et al., 2015), it suggests that HK Spn does affect the PER2 expression in mouse 3 cells (Figure 5C) even though it is a small effect. Hence, this could be because there were not enough bacterial stimuli to trigger the response in these different cell lines. Therefore, this led to a hypothesis that the bacterially induced PER2 response is perhaps a dose-dependent effect.

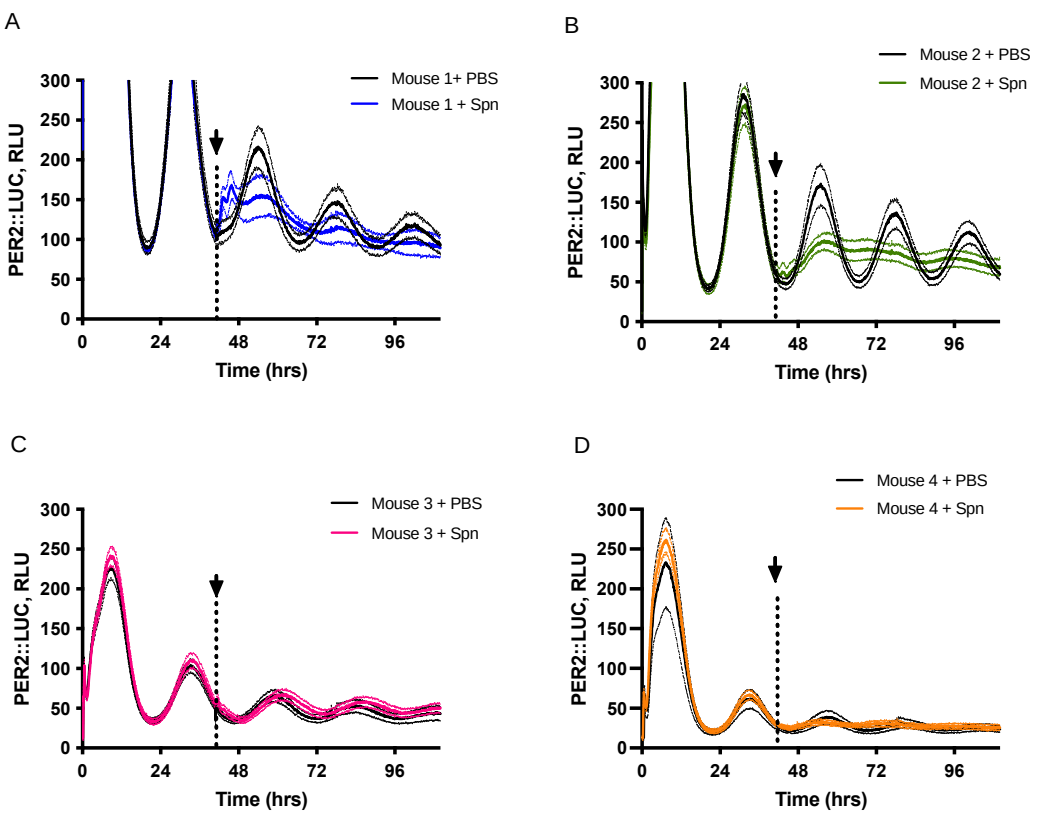
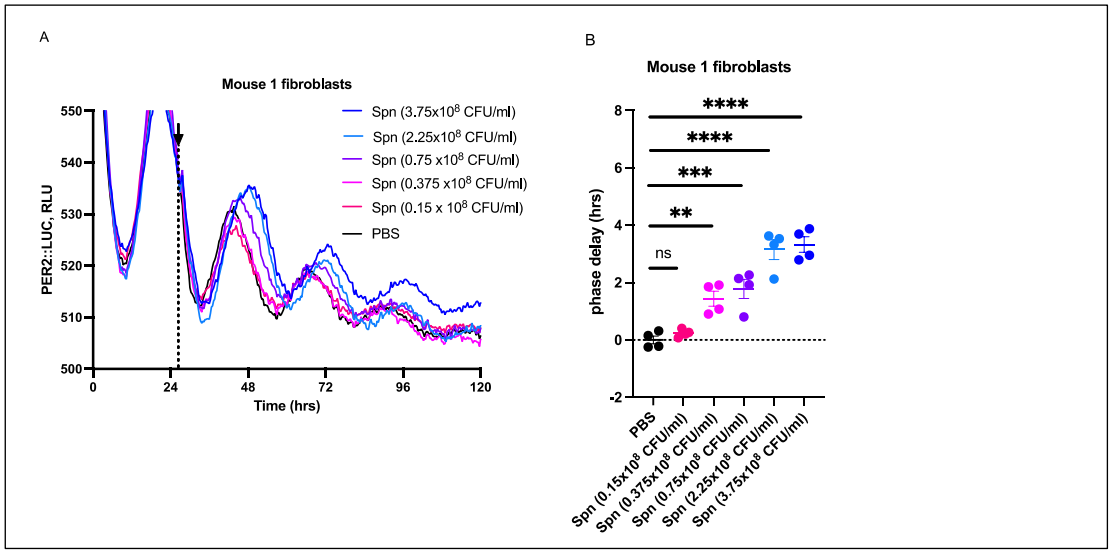


Figure 5. PER2 expression following HK Spn addition in multiple cell lines from different PER2::LUC mice. A). Lung fibroblasts from mouse 1. B). Lung fibroblasts from mouse 2. C). Lung fibroblasts from mouse 3. D). Lung fibroblasts from mouse 4. The dotted line denotes when PBS or HK Spn (0.75 x10^8 CFU/ml) is added. Technical replicates n≥3. Traces are shown as mean ± SEM

To investigate whether a higher concentration of bacterial addition affects the rhythms, different doses of HK Spn were tested on mouse 1 cells and mouse 3 cells because these two cell lines have opposite phenotypes following the bacterial addition. Acute induction of PER2 and phase shifting are observed in mouse 1 cells; on the other hand, no induction of PER2 and minimal phase shifting are observed in mouse 3 cells. Figures 6A and 6B show that the bacterially induced PER2 response in mouse 1 cells depends on the dose of HK Spn. For instance, cells exposed to the highest concentration

of HK Spn ($3.75x10^8$ CFU/ml) present a more extensive phase-shifting than at the other concentrations (Fig. 6A). Likewise, the PER2 response following bacterial addition in mouse 3 cells also depends on the concentration of HK Spn. Figures 6C and 6D indicate that at a higher concentration of HK Spn ($3.75x10^8$ CFU/ml), PER2 induction is observed. Therefore, this explains the absence of PER2 induction in the previous experiment where I only exposed these mouse 3 cells with a low concentration of HK Spn ($0.75x10^8$ CFU/ml). Thus, HK Spn can stimulate similar PER2 induction in mouse 3 cells as it is seen in mouse 1 cells.



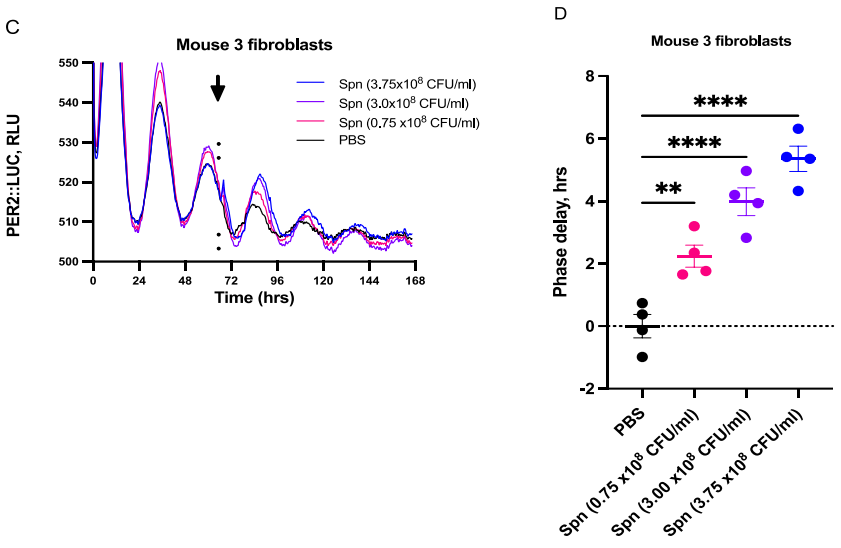


Figure 6. **PER2 expression following different concentrations of HK Spn addition in mouse 1 lung fibroblasts and mouse 3 lung fibroblasts.** A). Zoom in of raw traces of PER2 expression in mouse 1 fibroblasts. The dotted line denotes when PBS or HK Spn is added. Data were normalized at the treatment time. Technical replicates n≥3. Traces are shown as mean B). Dose response curve (DRC) of mouse 1 fibroblasts. X axis represents the concentrations of HK Spn in CFU/ml. Y axis represents the phase delays in hours C). Zoom in of raw traces of PER2 expression in mouse 3 fibroblasts. Data were normalized at the treatment time. Technical replicates n≥3. Traces are shown as mean ± D). Dose response curve (DRC) of mouse 3 fibroblasts.

3.2 PER2 response is conserved among various HK bacteria and HK yeast

Gram-positive bacteria have a thick peptidoglycan layer, while Gram-negative bacteria have a thin peptidoglycan layer (Takeuchi et al., 1999). Moreover, Gram-positive bacteria do not have an outer lipid membrane, yet Gram-negative bacteria do. These peptidoglycan layers from these two groups of bacteria are evolutionarily different, leading to varying motifs. Due to these differences in the peptidoglycan layers between Gram-positive and Gram-negative bacteria, specific immune recognition and responses are required to contain the infection (Takeuchi et al., 1999). For example, Gram-positive bacteria are recognized by lipoteichoic acid in the

membrane peptidoglycan layer; on the other hand, identifying Gram-negative bacteria involves the lipopolysaccharide on the outer membrane.

I wanted to know whether this PER2 response is specific to HK Spn or if other bacteria stimulate this induction. The PER2::LUC fibroblasts were exposed to various heat-killed bacteria, including Gram-positive bacteria (Spn, *Streptococcus agalactiae*, *Staphylococcus aureus)*, and Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*). Interestingly, all these HK bacteria induce PER2 (Figures 7A-D). From these bioluminescence traces of PER2, phase shifting is observed in all HK bacteria. The phase-shifting was quantified as the difference in phase between the average phase of PBS treated cells vs. HK bacteria treated cells (Figure 7C). However, there is a limitation in this experiment which is the short recording time of post-treatment; the time window that I selected to fit the raw data to the cosine equation is small. Thus, the phase values and amplitudes values that obtained from the fitted line might not be accurate.

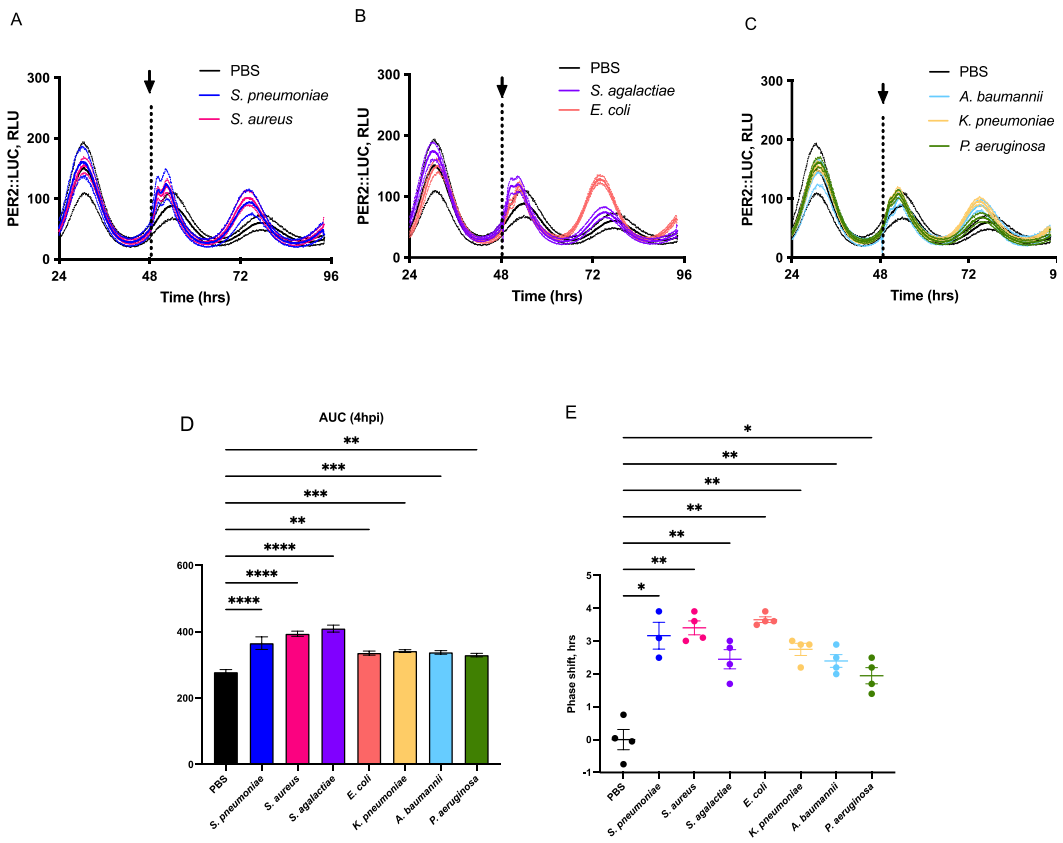


Figure 7. **PER2 expression following different HK bacteria in mouse 1 lung fibroblasts.** Zoom in of raw traces of PER2 expression in mouse 1 fibroblasts with various HK bacteria: A). *S. pneumoniae* (blue), *S. aureus* (pink) B). *S. agalactiae* (purple), *E. coli* (orange) C) *A. baumannii* (light blue), *K. pneumoniae* (yellow), *P. aeruginosa* (green). The dotted line denotes when PBS or HK bacteria ($0.75x10^8$ CFU/ml) is added. Technical replicates n≥3. Traces are shown as mean ± SEM D). Quantification of PER2 expression as area under the curve analysis at 4-hour post infection. Technical replicates n≥3. E). Quantification of phase shifting in hours. Phases were obtained from damped cosine equation. Phase shifting was measured by the differences in phase between the average of PBS treated group and each replicate of the bacteria-treated groups.

Therefore, a repeat experiment was done with a more extended post-treatment recording. These mouse lung fibroblasts were exposed to three HK bacteria: S. pneumoniae, *S. aureus*, and *E. coli*. Like HK Spn, these two HK bacteria also induce PER2 in mouse lung fibroblasts (Figure 8). Thus, this PER2 induction is an evolutionarily conserved bacterial response. Nonetheless, the analysis does not show

the phase-shifting to be significant in *S. pneumoniae* and *S. aureus*-treated cells. This was probably because the change in period (Figure 8D). Further analysis or normalization is needed. However, from the bioluminescence traces, these HK bacteria-treated cells are shifted compared to the PBS-treated group as bacteria-treated cells are almost in the opposite phase compared to the PBS-treated cells (Figure 8A).

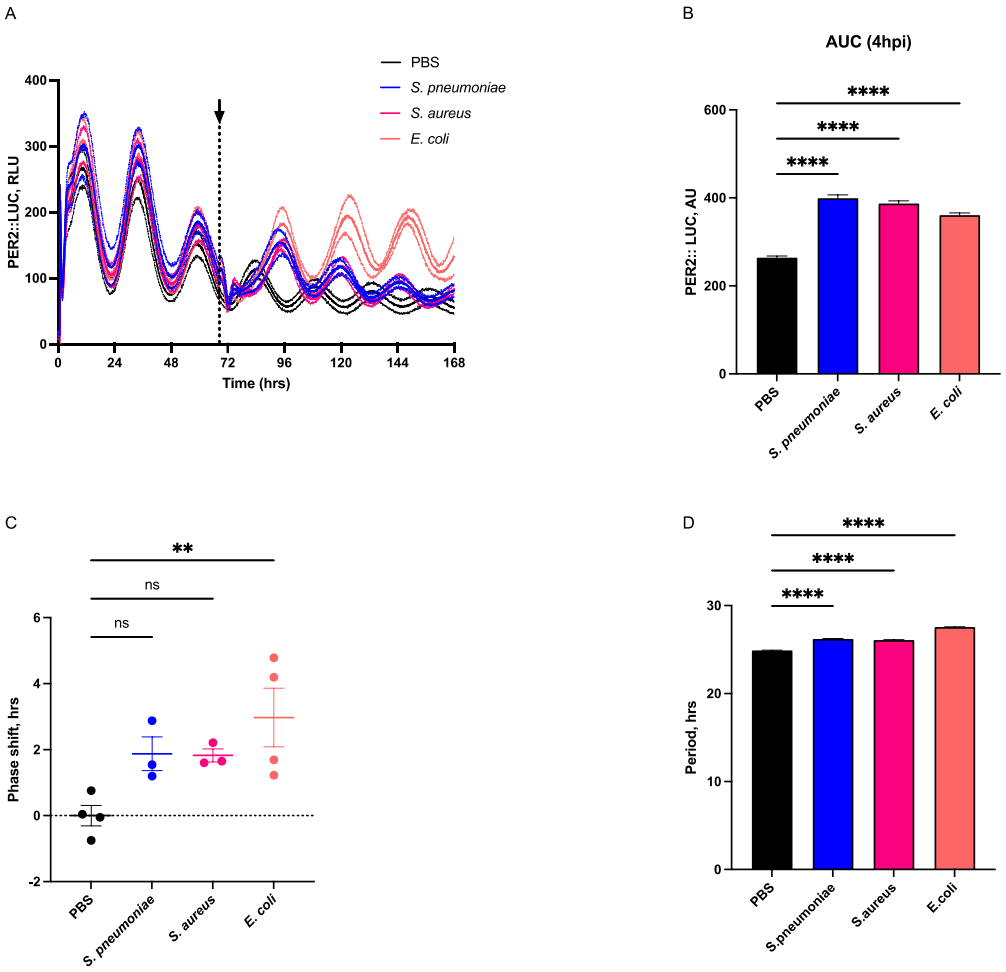


Figure 8. **PER2 expression following different HK bacteria in mouse 1 lung fibroblasts.** A). Raw traces of PER2 expression in mouse 1 fibroblasts. The dotted line denotes when PBS or HK bacteria ($0.75x10^8$ CFU/ml) is added. Technical replicates n≥3. Traces are shown as mean ± SEM B). Quantification of PER2 expression as area under the curve analysis at 4-hour post infection. C). Quantification of phase shifting in hours. Phases were obtained from damped cosine equation. Phase shifting was measured by the differences in phase between the average of PBS treated group and each replicate of the bacteria-treated groups. D). Quantification of period in hours.

Since all HK bacteria that I tested cause PER2 induction in mouse lung fibroblasts, I wanted to know if this response is specific to bacteria only or can it result from other microorganisms such as fungi. Hence, I exposed these cells to the HK yeast, *Saccharomyces cerevisiae*. Similarly, HK *S. cerevisiae* also induces PER2 (Figure 9A). The peak of induction for this experiment was not at 4 hours post-infection as it was in the investigation in Figure 3. The shape of the induction peak was different as the peak of induction was earlier. This could be due to the time of bacterial addition; cells were exposed to HK Spn at a different phase in oscillation compared to the phases they were exposed to HK Spn in Figure 3. However, to keep it consistent, the area under curve analysis was done from the infection to 4 hours post-infection; nonetheless, Figure 9B suggests that PER2 is increased in these treatment groups. The effect of phase-shifting from HK Spn and HK yeast is not significant in this experiment. One reason could be the change in period (Figure 9D); thus, further analysis or normalization is needed. Nonetheless, the bioluminescence traces present that HK bacteria-treated cells and HK yeast-treated cells have opposite phases than the PBS control group upon the stimulation (Figure 9A).

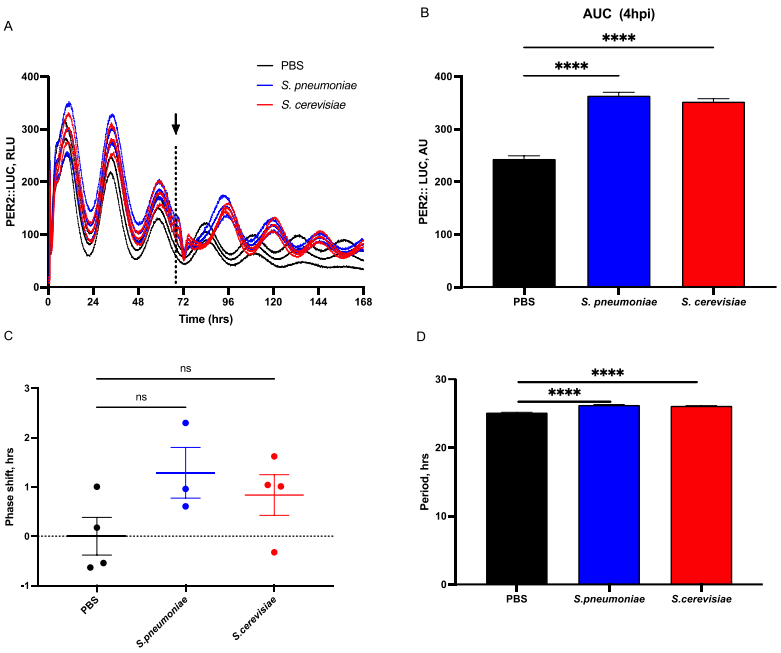


Figure 9. **PER2 expression following the addition of HK yeast *S. cerevisiae* in mouse 1 lung fibroblasts.** A). Raw traces of PER2 expression in mouse 1 fibroblasts. The dotted line denotes when PBS or HK Spn ($0.75x10^8$ CFU/ml) or HK *S. cerevisiae* is added. Technical replicates n≥3. Traces are shown as mean ± SEM B). Quantification of PER2 expression as area under the curve analysis at 4-hour post infection. Technical replicates n≥3. Traces are shown as mean ± SEM C). Quantification of phase shifting in hours. D). Quantification of period

3.3 Bacterially induced PER2 response is not mediated through TLRs

Given that a similar response is seen when PER2::LUC cells are stimulated with either HK bacteria or HK yeast, I wanted to test whether a component on these microorganisms' cell walls or membrane can induce PER2 response in these cells. Hence, I first experimented with lipoteichoic acid (LTA), an essential polymer in Gram-positive bacteria (Takeuchi et al., 1999). Moreover, LTA is a TLR2 ligand, thus elucidating whether PER2::LUC fibroblasts detect the bacterial component through the common TLR pathway. In this experiment, the LTA from *Bacillus subtilis* (*B. subtilis*) was used. Although I did not test with HK *B. subtilis*, previous experiments show that

the response is not specific to a particular bacterium; therefore, LTA from *B. subtilis* is a reasonable bacterial component to test. Figure 10A shows that LTA (30 µg/ml) does not increase PER2 expression. A zoom-in at exposure time (Figure 10B) also confirm the absence of PER2 induction peak in LTA-treated cells. Moreover, Figures 10C and 10D indicate that there is no effect on phase or period in cells that were exposed to LTA. Even though 10 µg/ml has been shown to activate TLR2 in HEK293 cells (Schwandner et al.,1999), 30 µg/ml has not been shown to activate TLR2 in mouse lung fibroblasts. Therefore, to confirm that LTA does not stimulate PER2 response, further experiments to find what concentration LTA can activate TLR2 in these lung fibroblasts are needed. Moreover, a positive control known to activate TLR2 in these cells is necessary to test this question. Downstream effector of the TLR2 pathway is NF-κB; thus, perhaps a NF- κB activator is a reasonable stimulus if we want to further investigate whether TLRs are involved in bacterially-induced PER2 induction

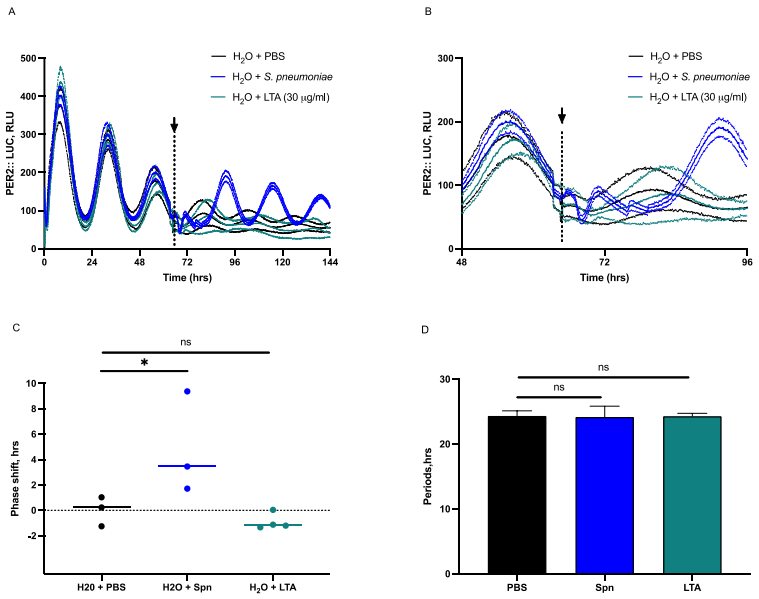


Figure 10. **PER2 expression following the addition of Lipoteichoic acid (LTA) in mouse 1 lung fibroblasts. A)** Raw traces of PER2 expression in mouse 1 fibroblasts. The dotted line denotes when PBS or HK Spn ($0.75x10^8$ CFU/ml) or LTA (30 µg/ml) is added. Technical replicates n≥3. Traces are shown as mean ± SEM . B) Zoom in of raw traces at the exposure time. C). Quantification of phase shifting D). Quantification of period.

Knowing that LTA does not induce PER2 expression, I wanted to test another TLR ligand, CpG DNA, a TLR9 ligand. The cytosine in CpG DNA remains unmethylated in the bacterial genome, yet CpG DNA is heavily methylated on the cytosine in the mammalian genome. Thus, bacterial CpG DNA is known as immunostimulant as it can stimulate an immune response in host cells (Ishii et al., 2006). Specifically, host cells can recognize foreign CpG DNA by TLR9 receptor. In this experiment, CpG DNA from Spn was extracted from overnight culture. The experiment was done first with CpG DNA at 2 concentrations: 10 µg/ml and 50 µg/ml. A concentration of 10µg/ml of CpG DNA has been shown to activate the TLR9 signaling pathway in murine fibroblasts (Meneghin et al., 2008). Figure 11 shows that

a high concentration of CpG DNA does not induce PER2; it seems to decrease PER2 expression instead. In addition, 10µg/ml of CpG DNA does not stimulate PER2 induction, yet there is a phase shifting. This could be an artifact since the traces of the cells before treatment for this group were different than the other treatment groups. Furthermore, the extraction of CpG DNA was challenging as the DNA yield was low, even starting with a large batch of bacterial cultures. Further experiments with protocol optimization are needed to ensure higher concentration and confirm that CpG DNA does not stimulate PER2 induction in PER2::LUC mouse lung fibroblasts. Moreover, a positive control such as a NF- κB activator to see if the CpG DNA induced a response in these cells is needed. The zoom in of traces of PER2 expression at bacterial exposure in Figure 11B confirms that there is no induction peak in 2 groups of cells that were exposed to CpG DNA.

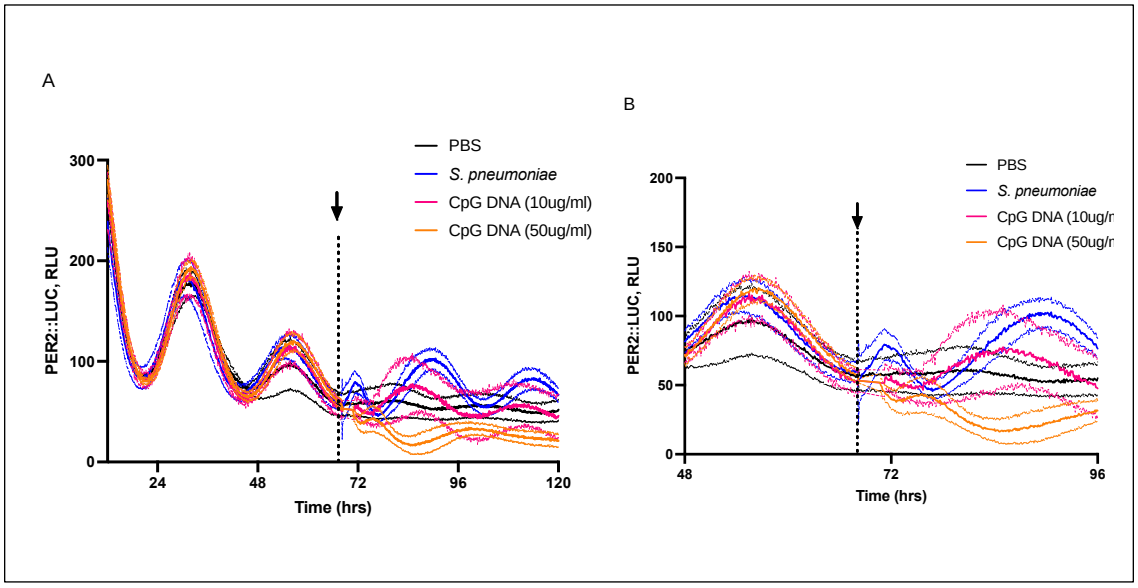


Figure 11. **PER2 expression following the addition of CpG DNA in mouse 1 lung fibroblasts.** A).Raw traces of PER2 expression in mouse 1 fibroblasts. The dotted line denotes when PBS or HK Spn ($0.75x10^8$ CFU/ml) or CpG DNA is added. Technical replicates n≥3. Traces are shown as mean ± SEM B). Zoom in of traces at the time of bacterial addition

Since both LTA and CpG DNA do not stimulate the PER2 response in these cells, I tested with another TLR ligand, lipopolysaccharide (LPS), a component of Gram-negative bacteria's outer membrane that is known to activate TLR4 receptor (Takeuchi et el., 1999). Figure 12A shows that LPS at concentrations of 800 ng/ml and 1400 ng/ml do not induce PER2 while HK Spn and HK *E. coli* induce PER2 induction and shift the phase of these cells. A zoom in at the bacterial exposure also confirm this observation (Figure 12B). Nevertheless, a positive control such as a NF- κB activator to see if LPS induced a response in these fibroblasts is needed.

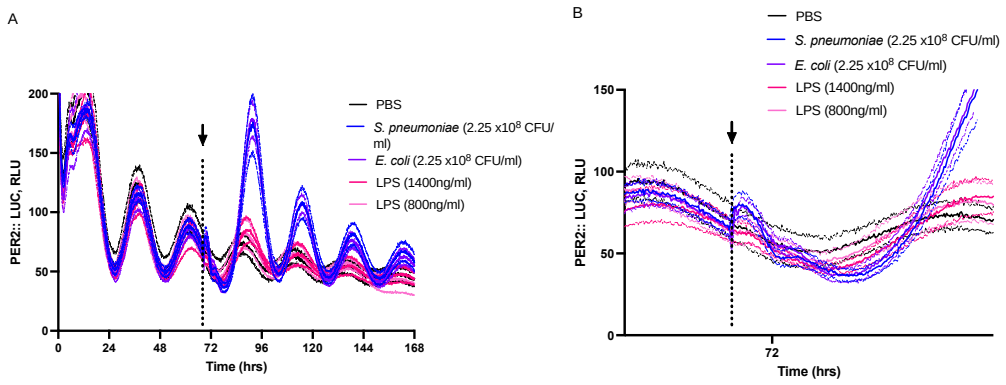


Figure 12. **PER2 expression following the addition of LPS (*E. coli*) in mouse 1 lung fibroblasts.** A) Raw traces of PER2 expression in mouse 1 fibroblasts. The dotted line denotes when PBS or HK Spn or HK *E. coli* or LPS is added. Technical replicates n≥3. Traces are shown as mean ± SEM. B) Zoom in of traces at the bacterial addition.

3.4 Bacterially induced PER2 response is not mediated through transcription

The increase in PER2 could be due to one or a combination of these reasons: (1) an increase in *Per2* mRNA, (2) an increase in protein without enhancing *Per2* mRNA level, or (3) a decrease in protein degradation. Therefore, I wanted to know whether an increase in PER2 protein was due to an increase in *Per2* transcript or not. I collected samples at 4 hours post bacterial exposure because 4 hours was the peak of PER2 induction. My RT-qPCR data confirm no significant difference in *Per2* transcript at 4-hour post-treatment with PBS or Spn (Figure 13A). However, an increase in *Per2* mRNA level could have occurred before the 4-hour post-bacterial addition. Therefore, RT-qPCR analysis was done for samples that were collected at shorter incubation times: 1 hour, 2 hours, 3 hours, and 4 hours post addition, and we found that HK Spn does not increase *Per2* mRNA levels (Figure 13B). A limitation in this experiment was the biological variation between replicates. Moreover, there was a low yield of RNA products after the extraction and before cDNA synthesis.

Another test approach was used to investigate whether HK Spn induces *Per2* mRNA by exposing PER2::LUC fibroblasts to the transcription inhibitor α-amanitin (Wienland et al., 1991) 30 minutes before the bacterial addition. Figure 13C shows that PER2 induction persists in the presence of transcriptional inhibitor α-amanitin at a 30 µg/ml concentration. The area under the curve analysis also presents that the α-amanitin does not decrease bacterial-induced PER2 induction as there is no significant difference between the two groups of control-treated with Spn and α-amanitin treated with Spn (Figure 13D).

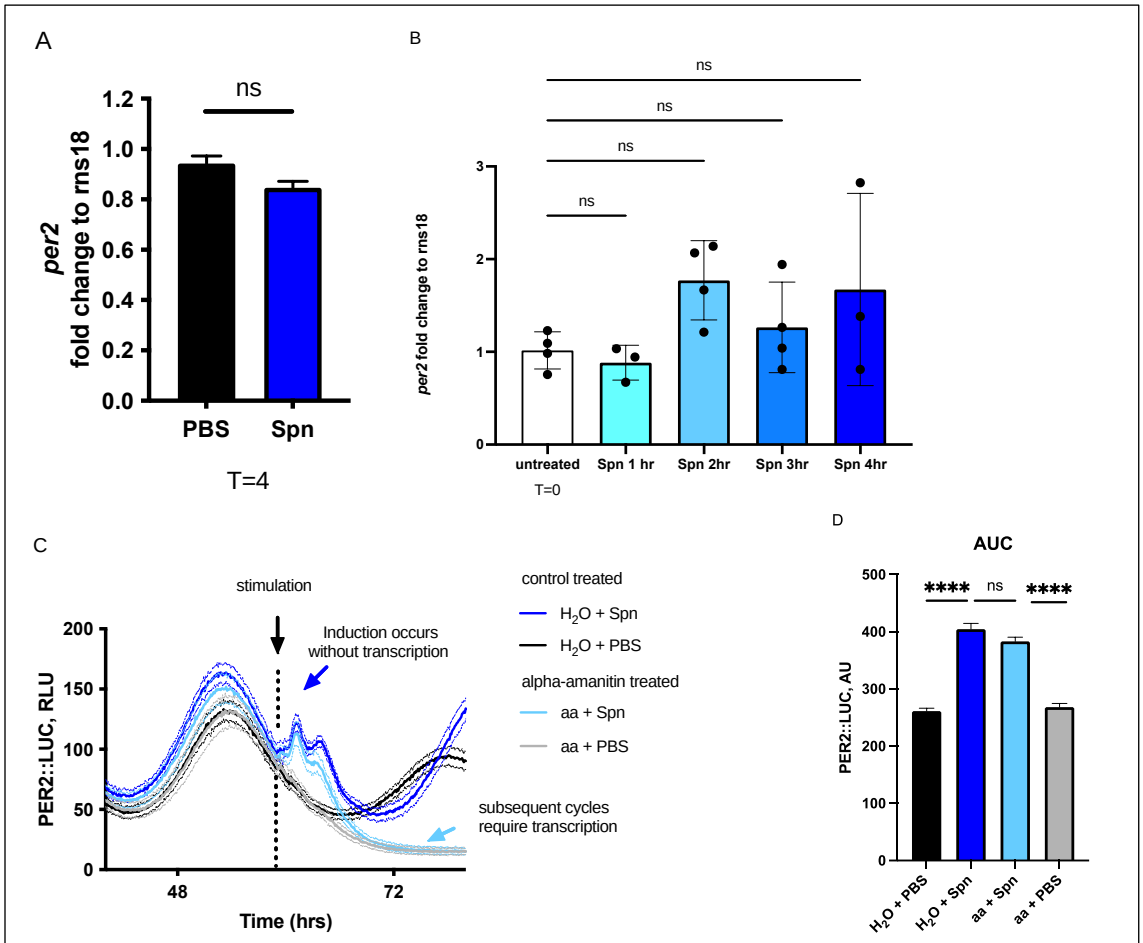


Figure 13. Acute PER2 induction by Spn is likely not mediated via transcription. A). per2 RT-qPCR analysis at 4hr post-stimulation with PBS or Spn ($0.75x10^8$ CFU/ml). B per2 RT-qPCR analysis at 1hour, 2 hours, 3 hours, 4 hours post-stimulation with untreated (T=0) or Spn. C). PER2::LUC fibroblasts treated with α-amanitin (inhibitor of RNA pol II and III) or control, then stimulated with PSB or Spn. This is representative data. Technical replicates n≥3. Traces are shown as mean ± SEM. D). Quantification of acute induction in the absence or presence of α-amanitin.

Furthermore, Leptomycin B, a nuclear export inhibitor that inhibits the transporting of proteins and mRNA between nucleus and cytoplasm (Watanabe et al. 1999), was also used to test whether the acute induction of PER2 following the treatment was due to transporting new transcripts. PER2 induction is not diminished in the presence of Leptomycin B (Figures 14A and 14B). These data suggest that the increase in PER2 expression is not due to the transport of new *Per2* transcript. Thus, as bacterial induction of PER2 persists in the absence of transcription (Figures 13C) or nuclear export (Figure 14A), and *Per2* transcript levels are not changed during induction (Figures 13A and 13B), we concluded the increase in PER2 protein is most likely due to increased translation of pre-existing cytoplasmic transcripts. We have not ruled out a contribution of altered protein stability. However, if the effect were mediated entirely by decreasing turnover, we would expect that PER2 traces would stabilize, leading to a broadened trace (as is observed when proteasome inhibitors are added in Crosby et al., 2019).

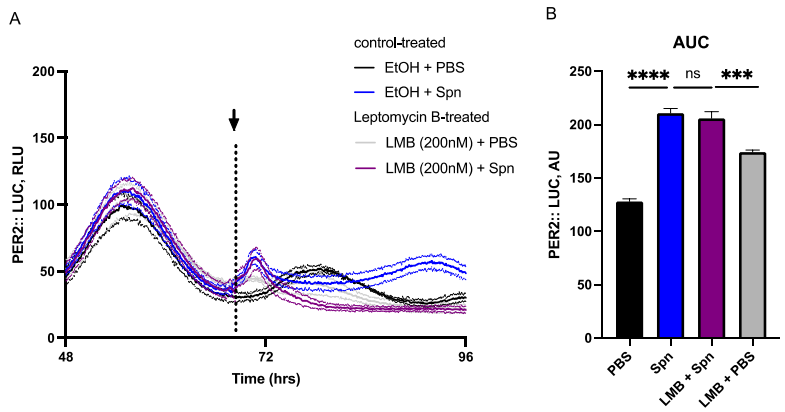


Figure 14. Acute PER2 induction by Spn is not mediated via transcription. A). Zoom in of raw traces of PER2::LUC fibroblasts treated with Leptomycin B (inhibitor of nuclear export) or control, then stimulated with PSB or Spn ($3.75x10^8$ CFU/ml). This is representative data. Technical replicates n≥3. Traces are shown as mean ± SEM. B). Quantification of acute induction in the absence or presence of Leptomycin B.

3.5 Bacterially induced PER2 response is not through the common translational pathway, mechanistic Target of Rapamycin (mTOR) pathways

Next, we investigated whether cellular pathways involved in translational regulation contribute to bacterially induced PER2 response. Two known stimuli affect the translation initiation of *Per2*: insulin (Crosby et al., 2019) and light (Cao et al., 2015). Insulin has been shown to increase PER2 translation by activating the mechanistic target of Rapamycin (mTOR) (Crosby et al., 2019), while Cao and colleagues showed that light affects PER2 translation in an mTOR-independent manner.

mTOR is a highly conserved protein kinase that regulates many cellular processes such as metabolism, translation, transcription, ribosome biogenesis, nutrient transport, and autophagy (Roux et al., 2018). Translation initiation is the most regulated step of the process. The formation of the initiation complex eIF4F is crucial. The three factors eIF4A, eIF4E, and eIF4G, need to form this complex. The rate-limiting factor is eIF4E; eIF4E binding protein (4E-BP) can bind to eIF4E, which prevents its association with the scaffolding protein eIF4G. Hence, the initiation complex is not formed; translation cannot start. Upon mitogenic stimulation, mTOR phosphorylates 4E-BP, which causes its dissociation from eIF4E. Thus, it allows the formation of the eIF4F complex and translation initiates (Roux et al.,2018).

First, we tested whether bacterially induced PER2 response is mTOR-mediated using the mTORC1 and mTORC2 inhibitor, Torin-1. Figure 15A shows that PER2 induction following the treatment of HK Spn is mTOR-independent, as Spn-mediated

PER2 induction persists in the presence of Torin-1. The area under curve analysis shows a not significant difference in PER2 expression between the control-treated Spn group (dark blue) and Torin-1 treated Spn group (light blue), as it is in Figure 15B. However, the area under the curve in the two control-treated groups is insignificant. This could be because of the low concentration of HK Spn ($0.75x10^8$ CFU/ml). Moreover, there is a decrease in bioluminescent signal in the Torin-1 treated cells as the Torin-1 + PBS treated group (gray) is also lower. Thus, it suggests that mTOR contributes to the translation of PER2. Furthermore, the decrease in the bioluminescent signal also affects the phase calculated from the damped cosine wave equation. Therefore, to analyze the phase shifting in this experiment, I measured the timing of the first circadian peak post-bacterial addition for each group. The phase-shifting in the Torin-1+ Spn treated group (light blue) is still the same as the phase-shifting observed in the PBS + Spn treated group (dark blue) as the timing of the first circadian peak in these two groups are similar (Figure 15C). Hence, the bacterially induced phase-shifting persists in the presence of Torin-1. mTOR inhibition does not inhibit the effect of HK Spn on the PER2 expression of these cells. Therefore, the PER2 induction that occurs post-transcriptionally is likely independent of mTOR. Nonetheless, a positive control to show that the Torin-1 is blocking mTOR-dependent signaling is needed. For example, the experiment could be repeated having 2 more conditions: insulin with and without Torin-1. The expected result would be that Torin-1 diminishes the PER2 induction by insulin but not the bacterially-induced PER2 induction.

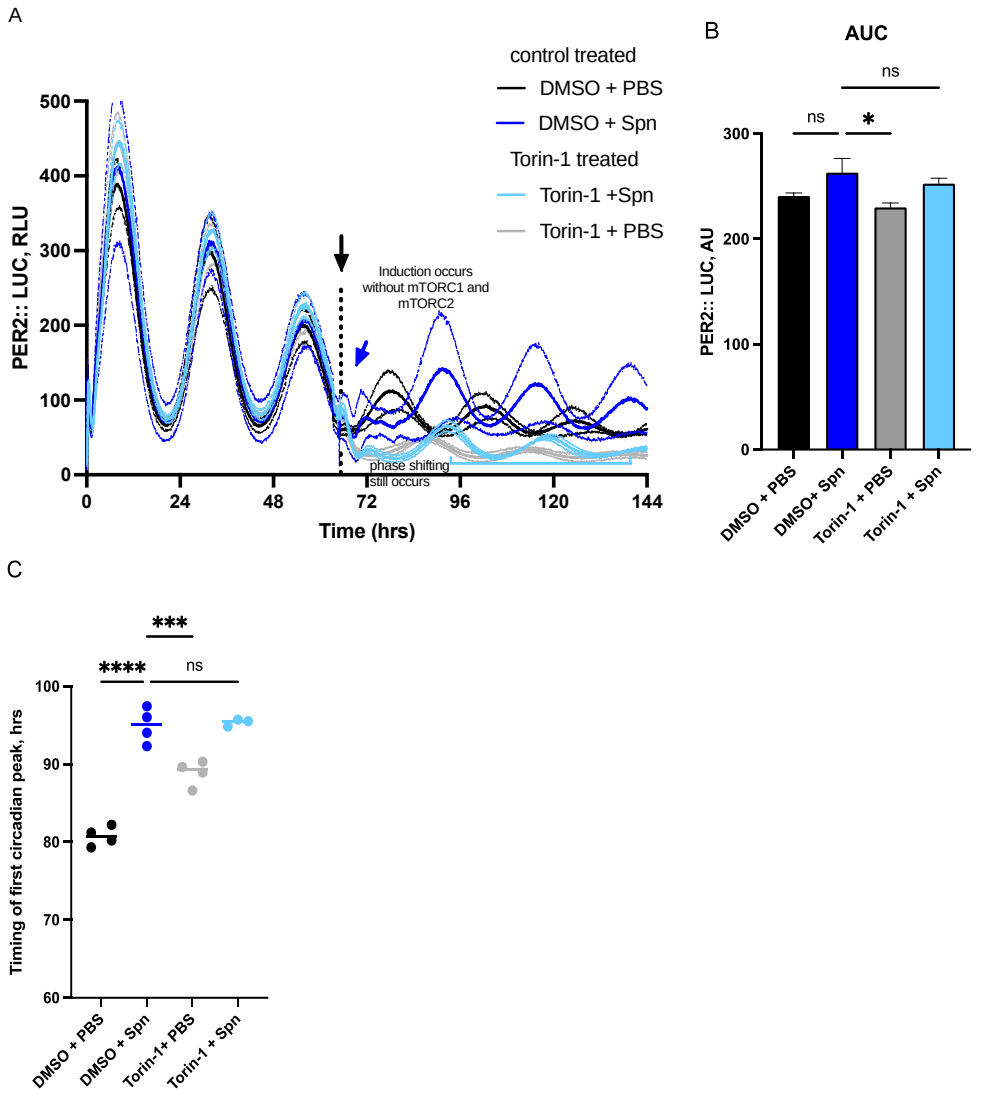


Figure 15. Acute induction of PER2 by Spn is not mediated via mTOR. A). Raw traces of PER2::LUC fibroblasts treated with Torin-1 (inhibitor of mTORC1 and mTORC2) or control, then stimulated with PSB or Spn. This is representative data. Technical replicates n≥3. Traces are shown as mean ± SEM. B). Quantification of acute induction in the absence and presence of Torin-1. C) Timing of first circadian peak post treatment in hours. Technical replicates n≥3

Chapter 4: Discussion

The circadian clock is an essential regulator of immune function as it regulates many aspects of immunity (Curtis et al., 2014; Tsoumtsa et al., 2016; Barik et al., 2019). Importantly, circadian rhythms have been shown to determine disease outcomes (Kitchen et al., 2020). However, the effects of bacterial infections on the circadian clock remain unknown. This project presents a novel relationship between the circadian clock and bacteria in which exposure to HK bacteria alters the phase and amplitude of PER2 oscillations without affecting the period. These three important features are discussed in more detail below.

Phase: HK Spn reset the clock of these mouse lung fibroblasts by increasing PER2 expression. It has been shown that a change in PER2 abundance is sufficient to shift the phase of these cells (D'Alessandro et al., 2015). Thus, due to this change in PER2 expression in these cells, phase shifting is observed. Furthermore, the phase-shifting following bacterial addition is found to be type 0 resetting as the resulting phase (CT10) is expected irrespective of the treatment time. This type of phase resetting is stronger than type 1 resetting, where the resulting phase depends on the treatment time. Moreover, it has been shown that the response can change from type 1 to type 0, resetting when the dose or strength of the stimulus is increased (Manella et al., 2021). Notably, the phase response curve from bacterial addition was done before the dose-response curve experiment. Thus, the phase response curve was not done with an optimal dose of bacteria, yet the effect from this experiment is type 0 resetting. Therefore, this work shows that bacterial exposure is a potent stimulus.

Importantly, studying the bacterially induced phase shifting to CT10, which corresponds to 2 hours after 32°C -37°C transition, would further elucidate how bacterial infection affects the clock. Cells have a constant daily oscillation in a lot of their functions. Thus, when a phase-shifting is observed, this will alter the timing of cellular processes. It would be interesting if we could determine whether HK bacteria shift cells to a clock phase where they are best fighting the infection or not. One suggestion is characterizing the differences in the production of antimicrobial-peptide in cells exposed to HK bacteria at CT10 with cells exposed at different times. Another approach is examining the barrier functions of fibroblasts exposed at CT10 and other times.

Amplitude: It has been shown that an increase in amplitude could be due to one of these two effects: (1) synchronization of the cell population or (2) a change in the rhythmicity of individual cells in the population (Izumo et al., 2006). Thus, we could see an increase in amplitude in these HK bacteria-treated cells because HK bacteria enhance the amplitude of every single cell in the population, or HK bacteria enhance the synchronization the cell population. It would be interesting if we could identify which one of these effects is the case. This could be done by monitoring responses at the single-cell level (Leise et al., 2012). Moreover, if the effect from HK bacteria is from individual cells instead of the cell population, studying whether affected cells have any influence on the rhythms of unaffected neighboring cells is expected to be of great value in the field.

Period: The bacterial stimulus does not affect the overall period (24-hour cycle). The difference in the period between cells treated with PBS and HK Spn was not significant in most experiments. In a couple of experiments, the period lengthening effect is seen in cells treated with PBS. This observation is similar to the past studies where the authors also reveal that the circadian period length varies among cells (Nagoshi et al., 2004; Leise et al.; 2012; Manelle et al., 2021). Thus, HK Spn induces PER2 but does not affect the overall period, indicating that HK bacterial addition can modify but not impair the clock.

Another interesting finding from this study is that bacterially induced PER2 response is likely not mediated through transcription. Previous studies have shown that most stimuli that affect circadian rhythms through the *Per2* gene are transcription. For example, these are transcriptional activators of PER2: glucocorticoids (Balsolobre et al.;2000a; Cheon et al., 2013), dexamethasone (Balsolobre et al. 2000b). On the other hand, glucose reset the circadian oscillation by decreasing *Per2* level (Hirota et al., 2002). Light exposure also resets the circadian clock in the SCN through *Per2* transcription (Challet et al., 2003). Thus, most known stimuli are through transcription of the gene. One known stimulus known to affect PER2 translation is insulin (Crosby et al., 2019). Moreover, Crosby and colleagues show that insulin affects PER2 through mTOR activation, PTEN inhibition, and downregulation of *Per2* cognate miRNAs (miRNA-24, 29, and 30). Nonetheless, this study found that bacterial-induced PER2 response does not go through the common translational mTOR pathway. Future studies are needed to find out how this response is mediated. miRNAs are good candidates, as

several groups have previously reported that miRNAs are involved in bacterial infections; for example, miRNA-24 expression is decreased in *S. aureus*-infected cells (Jin et al., 2015), and miRNA-155 is critical for the clearance of *S. pneumoniae* (Verschoor et al., 2014).

All in all, this study shows that HK Spn can reset the clock via induction of PER2. This effect can be seen in infection times throughout the clock to different extents, resulting in shifting the cells to CT10 regardless of what time these cells were exposed to the treatment. This response is in dose-dependent manner, and the response can be seen in other cell lines from different PER2::LUC mice.

Furthermore, we characterized that the PER2 response is an evolutionarily conserved response within multiple HK bacteria, including Gram-positive and Gram-negative bacteria. Thus, the response is not specific to HK Spn. HK yeast *S. cerevisiae* also can stimulate the PER2 response in these cells. LTA, LPS and CpG DNA do not seem to contribute to this response. Interestingly, bacterially induced PER2 response is not through transcription nor the common translational mTOR pathway. Therefore, this work elucidates new findings on how the bacterial infection itself affects the circadian clock. Yet, there are still a lot of exciting questions to be explored, such as whether HK bacteria affects the circadian clock in different cell types (heart, kidney, liver, gut). It would also be interesting to investigate how bacterial infection affects the clock in vivo.

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