

Journal of Visualized Experiments

The use of mouse splenocytes to assess pathogen-associated molecular pattern influence on clock gene expression --Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58022R1
Full Title:	The use of mouse splenocytes to assess pathogen-associated molecular pattern influence on clock gene expression
Keywords:	Molecular clock; Circadian rhythms; Lipopolysaccharide; ODN1826; Heat-killed <i>Listeria monocytogenes</i> ; Splenocytes; Toll-like receptors; Pattern-recognition receptors; Pathogen-associated molecular patterns; Immunology
Corresponding Author:	Adam C Silver University of Hartford West Hartford, CT UNITED STATES
Corresponding Author's Institution:	University of Hartford
Corresponding Author E-Mail:	asilver@hartford.edu
First Author:	Adam C Silver
Author Comments:	
Additional Information:	
Question	Response
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	

Dear Editor,

Thank you for the opportunity to revise the manuscript entitled, The use of mouse splenocytes to assess pathogen-associated molecular pattern influence on clock gene expression. I would also like to thank the reviewers for their positive feedback and insightful comments. I feel as though I have sufficiently addressed all the comments, which in the process, has significantly improved the manuscript. The responses to the comments can be found below. The improvements made are a large reflection on the peer review of this manuscript, so again, thank you.

Sincerely

Adam C. Silver

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

>>This was completed.

3. Please define all abbreviations before use.

>>This was completed.

4. Please use SI units, e.g. please use “ μL ” instead of “ μl ”, “ mL ” instead of “ ml ”. Please leave a white space between the values and the units.

>>The changes were made.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols ([™]), registered symbols ([®]), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently

referenced in the Table of Materials and Reagents. For example: “TaqMan”, “StepOnePlus”, “NanoDrop”, etc.

>>Commercial language was removed.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

>>This was completed.

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. The Protocol steps should contain only 2-3 actions per step and a maximum of 4 sentences per step.

>>This was completed.

8. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

>>This was completed.

9. For steps that involve software or analyzing tools, please make sure to provide all the details such as “click this”, “select that”, “observe this”, etc. Please mention all the steps that are necessary to execute the action item. Please provide details so a reader may replicate your analysis including buttons clicked, inputs, screenshots, etc. This is the level of detail we’re looking for. Please keep in mind that software steps without a graphical user interface cannot be filmed.

>>This was completed.

10. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

>>An ethics statement was provided.

11. Please include the age, sex and strain of the animals.

>>>The information was added.

12. Protocol: 1.1: Please include all the conditions for keeping the animals or refer to

appropriate references. Please define all abbreviations before use.

>>ZT was defined and a reference was inserted describing basic animal husbandry.

13. Protocol: 2.2: How much of each solution should be prepared? Please include the containers.

>>The information was added

14. Protocol: 2.3: How the culture media is warmed? Using what? How much PBS is needed? What is the pH of the buffer?

>>The information was added

15. Protocol: 2.4: Please include the pipette size.

>>The information was added

16. Protocol: 2.5: How much ethanol is needed?

>>The information was added

17. Protocol: 3.1: Please clearly describe the euthanasia process or refer to appropriate references. Please use the imperative tense for all the sentences in the protocol step. Please attention that euthanasia process can not be filmed, so please do not highlight this steps.

>>The highlight was removed. The following sentence was also removed, "For this particular experiment, splenocytes were isolated from mice that were sacrificed at ZT13" as the timing of sampling is described in the discussion. Details regarding the euthanasia process was added as was a reference.

18. Protocol: 3.2: How to cut away the fur? Using what?

>>This information was added.

19. Protocol: 3.3: Please use sub-steps for long protocol steps.

>>sub-steps were added

20. Protocol: 3.5: Please use the imperative tense for all the sentences in the protocol steps.

>>The step was reworded

21. Protocol: 3.7: How to transfer the medium?

>>This information was added

22. Protocol: 3.8: What is the temperature of the centrifugation?

>>This information was added.

23. Protocol: 4.1, 4.2: Please use the imperative tense for all the sentences in the protocol steps. Please avoid using any commercial language, please use a generic term instead.

>>The changes were made and a sub-step was added

24. Protocol: 4.3: The Protocol steps should contain only 2-3 actions per step and a maximum of 4 sentences per step. Please use sub-steps. Please clearly describe the actions in the imperative tense. Please include the pipette size and the containers.

>>The relevant information was included and the step was sectioned into sub-steps.

25. Protocol: 5.1, 5.2, 5.4: Please avoid using any commercial language (e.g., StepOnePlus, TaqMan, etc.), please use a generic term instead.

>>These terms were removed

26. Protocol: 6.1: Please use the imperative tense for all the sentences in the protocol steps. Please clearly describe the actions or refer to appropriate references.

>>The section was re-written

27. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file.

>>The table was removed

28. If you are reusing figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [AUTHOR] et al.[REFERENCE]".

>>This does not apply

29. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please list all the materials, equipment, instrument, and software used in your work.

>>The table has been modified

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

very nice summary of the relevance of the clock and what this technique will provide. Very detailed protocol and nice discussion of how to interpret the data

Major Concerns:

None

Minor Concerns:

None

Reviewer #2:

Manuscript Summary:

This paper describes a method to use mouse splenocytes to assess Pathogen-Associated Molecular Pattern Influence on clock gene expression. Over all, this paper is well written, and clearly stated.

Major Concerns:

1. In this paper, the author collected splenocytes from the tissue, and wanted to isolation the immune cells from the splenocytes. But in this method, the author did not detect the cell type he/she got, so it is not sure whether the cell in this paper is immune cells or not.

>>While I did not demonstrate the splenic cell composition in this method, the immune cell percentages present in the spleen have been previously determined and confirmed by my colleagues and I (unpublished data). The cell percentages are listed in the discussion. We used this splenocyte isolation protocol previously and subsequently worked with an adhere cell population: <https://www.heliyon.com/article/e00579>

2. In the paper, after separated the cell from the splenocytes, the author used RPMI 1640 with 10% FBS to incubate the cell. As we know, FBS can induce circadian gene expression in mammalian tissue culture cells (PMID:9635423), and the cultured cells.

So, the dissecting time does not have much meaning.

>>I have previously tried to trigger the clock *ex vivo* in splenocytes using the above method. However, I was unable to do so (unpublished data) and that was using 50% serum as was used in the paper (Balsalobre, 1998) mentioned by reviewer #2. If a serum shock using 50% serum did not reset the clock in these cells, then I do not believe 10% would.

>>More importantly, I have assessed expression of *Per2* and *Rev-erba* in splenocytes isolated over a 24-h period using the same method I am describing in this protocol. Daily changes in gene expression were observed (<http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0189949#sec013>), which demonstrates that the serum in the RPMI is not resetting the clock. If the clock was reset, expression would not change over the 24-h period.

Minor Concerns:

1. Line 44, it should make clear that the definition of the "master clock" in mammals or mice.

>>This information was added.

2. Line 82, "Animal Ethics Statement" should be stated.

>>An ethics statement was added

3. Line 82, Light intensity should give. Mouse age and gender should be made clear.

>>This information was added

4. Line 101, How long the time need for sterilize should give.

>>Scissors and forceps were originally autoclaved. The ethanol was used to prevent contamination and keep them 'clean.' This information was added to the protocol.

5. Line 105, "600 ml per sample", it is not clear the sample size, sample weight or cell number should give.

>>This information was added.

6. Line 157, it is better to give the RNA weight, not the volume.

>>In Protocol: 4.2 I added that up to 2 μ g can be used. Since every well contains 10^6 cells, the amount of RNA that is extracted is very consistent and yields under 2 μ g, therefore, I use 10 μ l of RNA during the cDNA synthesis step.

7. Line 181 and 187, again, cDNA weight should also give.

>>In Protocol: 5.2, I added the range of cDNA that can be used for the reaction.

8. Figure 1, the SD or SEM should be given in the figure, not only the mean value.

>>The SEMs were added to the figure.

Reviewer #3:

In this manuscript, Dr. Silver describes a protocol to measure the impact of PAMPs on clock gene expression in splenocytes. The protocol consists in harvesting spleens from mice, culturing the splenocytes in the presence or absence of LPS or other PAMPs, preparing RNA and using it for RT-qPCR for clock genes. Although the manuscript is generally clear and well written, and the protocol might be useful to some readers/viewers, it would benefit from the clarifications requested below.

Main concerns:

1) This protocol is extremely basic. Culture of primary splenocytes is routine use in numerous labs, as qPCR is too. I know that JoVE is fine with publishing even very basic approaches, so I guess that publishing this manuscript is relevant. I have not checked previous JoVE manuscripts/videos, but I would have assumed that there are already some that describe either splenocyte culture or qPCR, perhaps both together. The novelty here is to present a protocol that brings the two together, in the context of clock gene expression. In any case, this protocol and video might be of interest to JoVE readers, but I feel that the author should better explain the novelty of what is presented.

>>While addressing the editor's and reviewers' comments, I believe this was accomplished.

2) The manuscript (and presumably the video too) should better explain why it is important to study the effect of PAMPs and TLR activation on clock genes. Such an explanation is absent both from the introduction and the discussion. A sentence on line 77 says "since disruptions of circadian rhythms can lead to serious pathology" as a rationale for the proposed approach. However, the protocol only addresses the acute effects of PAMPs, not the (longer term) effect on clock function itself.

>>I believe the following will better explain the usefulness of this protocol.

>>The following sentence was added to the introduction: "The molecular clock has been shown to modulate various aspects of the immune response, therefore, disruption of the molecular clock would most likely impair the proper time-dependent variation of the immune response."

>>>The following paragraph was added to the discussion: Moving forward, while this protocol only addresses the acute effects on clock gene expression after PAMP challenge, it could provide proof of principle for further investigation. For example, this assay could be used as a model to decipher the molecular mechanisms regarding TLR

– PAMP interaction and how it influences the molecular clock. It could also be used to determine the length of time it takes for the molecular clock to recover after a PAMP challenge, which could be determined by conducting a time-course experiment (*i.e.*, assessing expression after varying times post challenge). As mentioned above, subsequent experiments could be performed to examine PAMP challenge on specific splenocyte cell populations. Since several pathogens stimulate multiple TLRs upon infection, it would be interesting to use this protocol to investigate if challenging with multiple PAMPs have a synergistic effect on clock gene expression.”

3) A general comment about the Protocol section of the manuscript is that although well written, I feel that it should be more detailed, and should describe which aspects are critical for it to work, including tips to optimize the procedure. Since this is a very basic protocol, it will be read (and watched) by people who presumably know little about splenocyte culture, qPCR or clock genes. Hence, no details should be omitted and no basic knowledge assumed as known by the readers.

>>While addressing the editor's and reviewers' comments, I believe this was accomplished.

4) The section on animals should be detailed. How are they entrained in the LD conditions, under which conditions exactly?

>>More details were provided.

It should also be explained that the procedure could be done under constant darkness if one would be interested in splenocytes under circadian free-running conditions.

>>The following was added to the discussion: “Diurnal rhythms are subjected to environmental stimuli (*e.g.*, light or food), which are termed zeitgebers. In the case of a 12-hr light / 12-hr dark cycle, the zeitgeber (*i.e.*, light) resets the clock to a 24-hr period. While most diurnal rhythms are circadian (*i.e.*, daily rhythms that occur in the absence of an external cue), they not true circadian rhythms until they have been shown to oscillate with an approximate 24-h period under constant environmental conditions. Therefore, this procedure could be performed using mice under constant conditions, which would entail entraining mice to the light-dark cycle as described above, but then the animals would be held in constant darkness for 3 days prior to sampling. This type of experiment is referred to as a dark-dark (DD) experiment and the time point of sampling would be referred to as CT (circadian time), not ZT.”

Even more important, the reason for the time of collection should be explained. Why harvesting the spleens at ZT13? This is not a time of day (evening) where most people would normally do their procedures, so explanations are needed.

>>The following was added to the first paragraph of the discussion: “Since *Dbp* and *Rev-erba* have been shown to demonstrate significant expression peaks in splenocytes and splenic immune cells around the light-dark interphase (ZT12), in the current method, cells were isolated and challenged at ZT13 in order to have a greater chance at detecting a reduction in these genes.”

Actually, based on what is already known in the literature about the circadian control of response to PAMPs, couldn't one expect that the response be different if spleens/splenocytes are taken at different times over the 24 h cycle? This would be important to mention, as a possible factor to consider (and test) in such experiments. (Or maybe this is not relevant, if the medium treatment resets the clock? See point 5, below.)

>>Yes, and this was addressed in the discussion: “When examining the impact of PAMPs on the molecular clock, the time of day when mice are sacrificed and splenocytes are subsequently challenged must be taken into consideration. *Tlr* expression and responsiveness has previously been shown to demonstrate time-of-day dependent variation^{9,23}, therefore, a time of day when TLR responsiveness is at its peak, could result in a greater influence on the clock. Furthermore, expression of molecular clock genes will also fluctuate throughout the day in splenocytes, therefore, a reduction of clock gene expression due to PAMP challenged would be most significant if examined during the time of peak expression¹⁰. Conversely, a PAMP that could increase clock expression, would most likely be observed if looking at a time of day when clock expression is at its lowest.”

5) I feel that the culture of the splenocytes and their stimulation is problematic when one considers that these cells have clocks which can be reset by serum treatment (like the one taking place when 3 ml of medium is added, line 134). Could this resetting by the serum mask the effect of PAMPs? For example, if a gene transcript is at high level in the cells, maybe one would not see a stimulation by a PAMP, but such an effect would be seen upon treatment at another clock time (time after synchronization of the cellular clocks). So maybe the protocol should include a wait of a few days after putting the splenocytes in culture (to let the clocks desynchronize) or conversely, take advantage of this clock synchronization and stimulate with PAMPs at different times after synchronization?

>>I have previously tried to trigger the clock *ex vivo* in splenocytes using the above method. However, I was unable to do so (unpublished data) and that was using 50% serum as was used in the paper (Balsalobre, 1998). If a serum shock using 50% serum did not reset the clock in these cells, then I do not believe 10% would.

>>More importantly, I have assessed expression of *Per2* and *Rev-erba* in splenocytes isolated over a 24-h period using the same method I am describing in this protocol. Daily changes in gene expression were observed (<http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0189949#sec013>),

which demonstrates that the serum in the RPMI is not resetting the clock. If the clock was reset, expression would not change over the 24-h period.

6) Why harvest cells 3 h after PAMP stimulation (line 136)? Is the time course the same for all PAMPs? And for all clock (or other target) genes? A time course (e.g. 1-2-3-6-12h) would be more advisable.

>>While a 3 h timepoint was chosen for this protocol, a time course experiment would be very interesting as it would allow one to determine the length of time it takes the clock to recover from PAMP challenge. I added this potential experiment to the discussion.

7) Why isn't the qPCR part planned to be in the video? It is not less complex nor less tricky than splenocyte preparation and culture (in fact, it is probably more, for someone not experienced in qPCR).

>>I would leave that decision up to the editor. From what I have seen, protocols in JoVE frequently have links to previously established protocols. I assumed this protocol would have a link to qPCR set up. However, I would be fine with including it in the video.

8) A problem with this protocol is the complex cell population that is studied, which limits the interpretation of the data. This is somewhat addressed in the Discussion, but it should be expanded, for example to explain how to interpret the data despite this limitation (e.g. based on the known expression of the TLRs in the spleen cells?) and how it could be solved experimentally (e.g. purification of the cell subsets prior to stimulation). I feel that this limitation is more problematic than the advantages listed for the protocol (large cell numbers, little tissue manipulation).

>>The following was added to the discussion: "For example, *Tlr9* expression rhythms in the mouse spleen differ between splenocytes, macrophages, B cells, and DCs¹⁶. Additionally, *Tlr1*, *Tlr3*, *Tlr4*, *Tlr6*, *Tlr7*, and *Tlr8* displayed significant daily oscillations in an adherent splenocyte population but only *Tlr2* and *Tlr6* experience daily oscillations in enriched splenic macrophages²⁵. Therefore, in order to investigate the outcome of a challenge on individual cell types, cells could be isolated via magnetic cell sorting, as previously described^{10,16} and then subsequently challenged."

>>Additionally, this protocol can be used for proof of principle for more expanded studies. This is stated in the revised concluding paragraph in the discussion.

Actually, the list of cells (line 259) is not correct: among the splenocytes obtained by the technique as described, there would be no DCs or macrophages, which would require collagenase treatment to be released from the tissue; this needs to be corrected. Related to this is the point made by the author about the circadian rhythm of these cell

types within the spleen: what could be the solution for this? Discuss.

>>I have previously used this isolation procedure and was able to obtain both macrophages/monocytes and dendritic cells:
<https://doi.org/10.1016/j.heliyon.2018.e00579>

>>Keller et al. use a similar approach as to what I describe (i.e., no collagenase treatment) to isolate CD11b+ cells.

>>Also listed below is a reference for a protocol comparing DC isolation via cell strainer vs. collagenase treatment.

>>While DC and macrophage numbers are lower without collagenase, it is incorrect to say that DCs and macrophages would not be present.

A circadian clock in macrophages controls inflammatory immune responses
Maren Keller, Jeannine Mazuch, Ute Abraham, Gina D. Eom, Erik D. Herzog, Hans-Dieter Volk, Achim Kramer, Bert Maier
Proceedings of the National Academy of Sciences Dec 2009, 106 (50) 21407-21412;
DOI: 10.1073/pnas.0906361106

Isolation of Mouse Spleen Dendritic Cells Andrew J. Stagg, Fiona Burke, Suzanne Hill, and Stella C. Knight From: Methods in Molecular Medicine, vol. 64: Dendritic Cell Protocols
Edited by: S. P. Robinson and A. J. Stagg © 2001 Humana Press Inc., Totowa, NJ

More specific comments:

- Line 3-4: The title should be revised to: "The use of mouse splenocytes to assess pathogen-associated molecular pattern influence on clock gene expression". (first few words revised to read better; and "molecular clock expression" — somewhat meaningless — revised to "clock gene expression")

>>The change was made

- Line 54: "Functional molecular clocks have been described..." In 2 of the 3 references cited here (Keller et al being the exception), only clock gene expression has been described. This is not sufficient to conclude that a "functional clock" is present in those cells. Assays to assess the cell-autonomous rhythmic gene expression or circadian function must be performed (e.g. rhythms in ex vivo culture) to conclude that there is a functional clock. Revise the sentence or change the references.

>>I removed one of the references. However, the Silver et al reference assessed clock gene expression under constant conditions in ex vivo splenic immune cells, which is what the reviewer is requesting (i.e., "rhythmic gene expression in ex vivo culture").

Therefore, I am confused as to why this reference would not be appropriate. The authors of this paper state, "Our data demonstrate that macrophages, DCs, and B cells enriched from mouse spleen possess functional molecular clocks as demonstrated by the daily oscillations in clock gene expression."

- Line 86: Define ZT at first occurrence.

>>The change was made.

- Lines, 97, 99, 118, and other occurrences: singular is "medium", not "media".

>>The changes were made.

- Line 105 (and 145): What is RLT buffer?

>>The following was added to the first place the buffer was used: "... Buffer RLT (a proprietary component of the RNA extraction kit that supports the binding of RNA to the silica membrane)..."

- Line 115: Cut only the fur, not also the tissue under it? And "left side of the mouse" is unclear: from which standpoint?

>>The following was added: "Using forceps, grab the peritoneum and carefully make an incision as not to damage the spleen."

>>"left side of the mouse" was changed to, "mouse's left side"

- Line 122: "between two sterile frosted slides": Mention that the tissue is grinded between the frosted parts. This is unclear. Also mention that the tissue and cells should remain wet and in the buffer at all times.

>>This information was added.

- Line 150: Briefly describe those manufacturer's instructions? Are there aspects of this manufacturer's protocol that are particularly tricky or that should be performed in a certain way?

>>While initially writing this protocol I asked the science editor about this issue. She told me that writing "according to manufacturer's instructions" would suffice.

- Line 154-155: Explain Nanodrop quantification and what the optimal range is. Also explain how to assess the purity of the RNA.

>>The following was added to the discussion: Within this protocol, a microvolume spectrophotometer can be used to quantify and assess the purity of the RNA being used in

determining gene expression. Nucleic acids absorb UV light at 260 nm, proteins typically absorb light at 280 nm, while other potential contaminants used during an RNA extraction procedure (e.g. phenol) are detected at 230 nm. Therefore, by assessing the absorbance (A) ratio at 260/280 nm (RNA to protein) and A260/230 (RNA to non-protein contaminants) the quality of the RNA can be assessed. High quality RNA has an A260/280 ratio between 1.8 – 2.1, as lower ratios indicate protein contamination. A pure RNA sample will have an A260/230 ratio of 2.0.

>>The range of RNA used in the cDNA synthesis was added to the protocol.

- Line 191: Why use actin as the control? Explain that any gene used as control in the qPCRs must be verified to ensure that it does not vary with the treatment studied. Explain how.

>> The following paragraph was added, “When determining the relative expression of a target gene (i.e., *Per2*, *Clock*, *Rev-erba*, and *Dbp*), an endogenous control gene (a gene in which expression levels do not differ between samples) must also be selected. Relative expression of the target gene is then normalized to the expression of the endogenous control gene. Differences in starting material (number of splenocytes), variation in reverse-transcriptase efficiency, varying rates of RNA degradation, etc., will be corrected for by the endogenous control gene (*β-actin* in this protocol). However, it is wise to verify that the treatment being examined does not alter expression of the endogenous control gene. This can be accomplished by assessing *β-actin* levels from several replicates of an equal amount of cells (treatment vs. non-treatment). In theory, their *β-actin* levels should be identical. Another approach to guard against endogenous control variation would be to use a panel of endogenous controls (e.g., *β-actin*, *Gapdh*, and 18S rRNA gene).”

- Lines 193-195: This data analysis point is very vague. A well validated approach such as the 2(-ddCt) must be used. Explain how. (See Livak KJ, Schmittgen TD. 2001. Methods 25:402-408.)

>>I have previously used this method:

Silver et al., The circadian clock controls toll-like receptor 9-mediated innate and adaptive immunity. *Immunity*, 2012

Silver et al., Circadian expression of clock genes in mouse macrophages, dendritic cells, and B cells. *Brain, Behavior, and Immunity*, 2012.

- Line 199: Use present tense as this is in the protocol ("is used").

>>This was changed.

- Line 263: Explain why it is essential to minimize animal and cell manipulations in clock studies.

>>The following was added: “because as mentioned above, these actions can disrupt the timing of the clock as well as clock controlled genes.”

- Lines 264-265: A n=3 is low, and could be insufficient depending on the stimulus used or the gene tested. Actually, the data provided in this manuscript suggest that there might be an effect of ODN1826 and HKLM on *Reverba* that does not reach significance due to a lack of power. If true, these data would argue for using a higher group size.

>>The following as added to the discussion: “It should be noted that increasing the number of animals per group might have revealed statistically significant differences between a challenge group and control (e.g., ODN 1826 and *Rev-erba*).

- Lines 266-268: The sentence starts by "Moving forward" but the rest is just to state the primary objective of this manuscript. The manuscript would gain from ending on a description of other possible applications of this protocol, beyond PAMPs, beyond clock genes, and perhaps with more conditions and time points.

>>I added a paragraph in the discussion that addresses these points.

- Line 262: Reference 20 is incomplete.

>>The reference was fixed.

Reviewer #4:

Manuscript Summary:

This work introduced an method to analysis the molecular clock genes expression in mouse splenocytes after challenged ex vivo with the pathogen-associated molecular patterns (PAMPs), lipopolysaccharide (LPS), ODN1826, and heat-killed *Listeria monocytogenes*. Overall, from my impression, this work is well done indeed. It introduced detailed procedures about entrainment of animals, instruments and reagents preparation, mouse splenocyte isolation and challenge, RNA isolation and cDNA synthesis, Quantitative PCR, and Statistical analysis. People who are interested to investigate the pathogens influence on splenocyte molecular clock work can easily followed the protocols to achieve their research targets. Therefore, I recommend to publish this manuscript without any hesitate.

Major Concerns:

No

Minor Concerns:
No

TITLE:

The Use of Mouse Splenocytes to Assess Pathogen-Associated Molecular Pattern Influence on Clock Gene Expression

AUTHORS AND AFFILIATIONS:

Adam C. Silver¹

¹Department of Biology, University of Hartford, West Hartford, CT, USA

Corresponding Author:

Adam C. Silver

asilver@hartford.edu

Tel: (860)-768-4587

KEYWORDS:

Molecular clock, Circadian rhythms, Lipopolysaccharide, ODN1826, Heat-killed *Listeria monocytogenes*, Splenocytes, Toll-like receptors, Pattern-recognition receptors, Pathogen-associated molecular patterns, Immunology

SUMMARY:

This protocol describes a technique using mouse splenocytes to discover pathogen-associated molecular patterns that alter molecular clock gene expression.

ABSTRACT:

From behavior to gene expression, circadian rhythms regulate nearly all aspects of physiology. Here, we present a methodology to challenge mouse splenocytes with the pathogen-associated molecular patterns (PAMPs) lipopolysaccharide (LPS), ODN1826, and heat-killed *Listeria monocytogenes* and examine their effect on the molecular circadian clock. Previously, studies have focused on examining the influence of LPS on the molecular clock using a variety of *in vivo* and *ex vivo* approaches from an assortment of models (*e.g.*, mouse, rat, and human). This protocol describes the isolation and challenge of splenocytes, as well as the methodology to assess clock gene expression post-challenge via quantitative PCR. This approach can be used to assess not only the influence of microbial components on the molecular clock but other molecules as well that may alter expression of the clock. This approach could be utilized to tease apart the molecular mechanism of how PAMP-Toll-like receptor interaction influences clock expression.

INTRODUCTION:

The master clock in mammals, which orchestrates 24-h oscillations for nearly all aspects of physiology and behavior, is located within the suprachiasmatic nucleus (SCN) of the hypothalamus^{1,2}. In addition to regulating biological processes on an organismal level, the master clock also synchronizes peripheral cellular clocks throughout the body³⁻⁵. While the molecular clock machinery consists of at least three interlocking transcriptional-translational feedback loops, the core is comprised of the *Period* (*Per1-3*), *Cryptochrome* (*Cry1-2*), *Bmal1*, and *Clock* genes^{6,7}. Besides maintaining the accurate timing of the core molecular clock, some ancillary

clock gene products (*e.g.*, *Rev-erba* and *Dbp*) also regulate expression of non-clock genes, *i.e.*, clock controlled genes (CCGs)^{6,7}.

Functional molecular clocks have been described in various immune tissues (*e.g.*, spleen and lymph nodes)⁸ and cells (*e.g.*, B cells, dendritic cells, macrophages)^{8,9}. These cells detect and respond to pathogen-associated molecular patterns (PAMPs), conserved microbial components, via innate immune recognition receptors such as Toll-like receptors (TLRs)¹⁰. To date, 13 functional TLRs have been described, which recognize microbial constituents such as bacterial cell wall components, flagellar protein, and microbial nucleic acids¹⁰. The PAMP, lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria recognized by TLR4, has been shown to alter circadian rhythms at the both organismal and molecular levels. For example, *in vivo* challenge of LPS induced photic-like phase delays as measured by activity in mice¹¹ and led to reduced clock gene expression in the SCN and liver as determined by *in situ* hybridization and quantitative PCR, respectively, in rats¹². After an *in vivo* challenge with LPS, analysis of human peripheral blood leukocytes¹³ and subcutaneous adipose tissue¹⁴ revealed altered expression of several clock genes as measured via qPCR. Lastly, *ex vivo* LPS challenges of human macrophages and mouse peritoneal macrophages, also led to altered clock expression as measured by qPCR¹⁴.

Here, we describe a protocol to assess the influence of the PAMPs LPS, ODN1826 (synthetic oligonucleotides containing unmethylated CpG motifs), and heat-killed *Listeria monocytogenes* (HKLM), recognized by TLR4, TLR9, and TLR2, respectively, on molecular clock gene expression in mouse splenocytes. The protocol includes mouse splenectomy, splenocyte isolation and challenge, RNA extraction, cDNA synthesis, and qPCR to assess expression of several clock genes. This protocol allows for the timely acquisition of a large number of immune cells with very little animal or cellular manipulation, which can then be challenged *ex vivo* with various PAMPs. The molecular clock has been shown to modulate various aspects of the immune response^{8,15,16}, therefore, disruption of the molecular clock would most likely impair the proper time-dependent variation of the immune response. In addition, since disruptions of circadian rhythms can lead to serious pathologies^{17–20}, it may be of interest for researchers to challenge splenocytes with a wide range of molecules and assess their influence on the clock.

PROTOCOL:

During the study, animal care and treatment complied with National Institutes of Health policy, were in accordance with institutional guidelines, and were approved by the University of Hartford Animal Institutional Animal Care and Use Committee.

1. Entrainment of Animals

Note: Twenty-week-old male B6129SF2/J mice are used in the study.

1.1) Entrain mice to a 12 h light (standard overhead white light) / 12 h dark cycle for 2 weeks prior to the experiment.

Note: Here zeitgeber time (ZT) 0 corresponds to lights on and ZT12 to lights off, while keeping all other environmental factors (*i.e.*, food, water, and room temperature) constant ²¹.

2. Preparation of Instruments, Culture Medium, and Challenge Medium

2.1) Autoclave forceps and dissecting scissors. Wrap pairs of frosted microscope slides in aluminum foil and autoclave.

2.2) Prepare culture medium by adding fetal bovine serum (FBS) to RPMI 1640 to a final concentration of 10%. Prepare 10 mL of challenge medium in 50-mL tubes by adding the following PAMPs to the culture medium (RPMI with 10% FBS); LPS (5 µg/mL), ODN1826 (5 µg/mL), heat-killed *Listeria monocytogenes* (10⁸ HKLM/mL), or another PAMP in its suggested concentration.

2.3) Warm culture medium, challenge medium, and approximately 70 mL of sterile phosphate buffered saline (PBS, pH 7.2) to 37 °C in a water bath.

2.4) Add 10 mL of culture medium using a 10-mL pipette and pipette aid to a 50-mL tube and place on ice.

2.5) Add approximately 30 mL of 70% ethanol to a 100-mL beaker and place ends of dissecting scissors and forceps into the beaker to prevent microbial contamination.

2.6) Prepare lysis buffer for RNA isolation by adding 10 µL β-Mercaptoethanol (under a fume hood) to every 1 mL of Buffer RLT in a 50-mL tube. Make only the amount that will be needed (600 µL per sample, which consists of approximately 1 x 10⁶ cells).

Note: The Buffer RLT is a component of the RNA extraction kit that supports the binding of RNA to the silica membrane.

3. Splenocyte Isolation and Challenge

3.1) Euthanize mice at a particular zeitgeber time via narcosis by keeping them in their original cage and adding CO₂ to the cage at a flow rate of 3 L/min. Continue supplying CO₂ for 1 min after breathing stops.

3.2) Confirm death via cervical dislocation by placing the thumb and index finger on either side of the neck at the base of the skull. Alternatively, press a rod at the base of the skull while quickly pulling (using the other hand) the base of the tail or the hind limbs to cause separation of the cervical vertebrae from the skull²².

3.3) Spray the mouse trunk with 70% ethanol and wipe with a paper towel. Place the mouse on its back and slightly tilted onto its right side. Cut away the fur, using dissecting scissors, along the mouse's left side, about halfway between the front and back legs.

3.4) Using forceps, grab the peritoneum and carefully make an incision so as not to damage the spleen. Remove spleen with forceps and place into a sterile 50-mL tube containing approximately 10 mL of culture media on ice. Repeat for the remaining animals.

Note: The spleen is the color of a kidney bean, and it is longer and flatter than the kidney.

3.5) Transfer one spleen with 2 mL of culture medium to a small sterile Petri dish.

3.5.1) Homogenize the spleen by grinding it between the frosted portion of two sterile frosted slides. Keep the issue and cells in the medium during the homogenization process.

3.5.2) Once thoroughly homogenized, pipet the 2 mL of culture medium containing the splenocytes through a 40- μ m nylon cell strainer into a 50-mL tube.

3.5.3) Repeat the above steps for the remaining spleens using a new pairs of frosted slides, 50-mL tubes with culture medium, and cell strainers.

3.6) Add 8 mL of cold culture medium to each of the 50-mL tubes containing the splenocytes for a total volume of 10 mL/tube. Determine the number of cells per milliliter using a hemocytometer.

3.7) Add approximately 1×10^6 cells/well to 6-well culture plates. Add cells to the number of wells that correspond to the number of different PAMPs being used for the experiment and include a control well. Add 3 mL of culture medium or 3 mL of challenge medium to the respective wells.

3.8) Incubate the plates at 37 °C in 5% CO₂ for 3 h.

3.9) Scrape the cells from the bottom of the well using a 1000 μ L pipet tip attached to a P1000 micropipette. Transfer the medium containing the cells using the same 1000- μ L pipet tip to a 15-mL tube.

3.10) Pellet the cells via centrifugation at 167 x g for 5 min at room temperature. Remove the supernatant and wash the cell pellet with 5 mL of PBS.

3.9) Pellet the cells a second time at 167 x g for 5 min, remove the supernatant, and add 600 μ L of lysis buffer to the cell pellet in order to lyse the cells. Then proceed to RNA isolation.

4. RNA Isolation and cDNA Synthesis

4.1) Isolate RNA from splenocytes using the RNA extraction kit according to manufacturer's instructions and perform the 'optional' on-column DNA digestion.

4.2) Prior to cDNA synthesis, determine RNA concentration using a microvolume spectrophotometer to verify that the concentration is within the optimal RNA range (up to 2 µg) of the cDNA synthesis kit.

4.3) Synthesize cDNA (to be used for the standard curve) for each of the samples using the reverse transcription kit according to manufacturer's instructions. Use 10 µL of RNA for each of the samples in a 20 µL total reaction volume.

4.3.1) Use a P100 or P200 micropipette to pool mRNA from a few control samples (*e.g.*, 5 µL from 2 samples for a total of 10 µL) into a 0.5-mL tube to prepare the cDNA.

4.3.2) Add 10 µL 2x reverse transcriptase master mix.

4.3.3) At the completion of the reverse transcription (thermocycler run), add 10 µL of H₂O to the reaction tube, which will serve as the starting concentration ("1") in the dilution series for the standard curve.

4.3.4) Perform a 10-fold dilution series (1 to 10⁻⁴) by adding 45 µL of water using a P100 or P200 micropipette into four 0.5-mL tubes designated 10⁻¹, 10⁻², 10⁻³, and 10⁻⁴.

4.3.4.1) Add 5 µL of the starting concentration "1" into the first tube (10⁻¹) using a P20 micropipettor, mix by pipetting up and down several times, then transfer 5 µL from the 10⁻¹ tube into the tube designated 10⁻² and mix.

4.3.4.2) Transfer 5 µL using a P20 micropipette from the 10⁻² tube into the tube designated 10⁻³ and mix. Transfer 5 µL using a P20 micropipettor from the 10⁻³ tube into the tube designated 10⁻⁴ and mix.

5. Quantitative Polymerase Chain Reaction (qPCR)

5.1) Determine relative quantitation of mRNA levels by qPCR. Within the experimental set up, select **Quantitation – Relative Standard Curve** and **TaqMan** chemistry. Change the reaction volume to 10 µL. Enter the relevant information into the plate layout (*e.g.*, target gene, standard curve, reporter, *etc.*).

5.2) Prepare the reaction so that it contains 0.5 µL of the primer/probe assay, 5 µL of gene expression master mix, 2 µL of H₂O and 2.5 µL of cDNA (10 – 100 ng).

5.2.1) Prepare a master mix by multiplying each of the preceding reagents (except the cDNA) by the number of reactions, making sure to include those from the standard curve, negative controls, performing each reaction in duplicate, and 2 extra reactions that will account for pipetting variation.

5.2.2) Pipet 7.5 μ L of the prepared master mix into pre-determined wells in a 96-well reaction plate with a micropipette or a multichannel pipette. Pipet 2.5 μ L of cDNA into the appropriate well for the unknowns and standards, and 2.5 μ L of H₂O into the negative control wells.

5.2.3) Include primer/probe assays for various molecular clock genes, and also include an assay for an endogenous control (e.g., β -actin).

5.5) In order to determine relative expression values, calculate the mean relative quantity for each replicate, then, for each sample, divide the mean relative quantity for the target gene by the mean relative quantity for β -actin.

6. Statistical analysis

6.1) Using statistical analysis software, enter data into the program under the column statistics option. Select a one-way ANOVA with the Dunnett's post hoc test to assess differences between mean values of clock gene expression after PAMP challenge versus the control.

REPRESENTATIVE RESULTS:

Mice were sacrificed at ZT13, splenocytes were isolated and challenged *ex vivo* with the PAMPs LPS, ODN1826, or HKLM. After 3 h, RNA was isolated, and qPCR was used to assess relative expression levels of the molecular clock genes *Clock*, *Per2*, *Dbp*, and *Rev-erba* compared to unchallenged control cells. After PAMP challenge, *Clock* expression levels were not significantly different than expression in the control cells (**Figure 1A**). *Per2* expression levels were significantly elevated in cells challenged with LPS and ODN1826 when compared to unchallenged controls (**Figure 1B**). LPS was the only PAMP to alter *Rev-erba* expression, as mRNA levels were significantly lower than in the unchallenged controls (**Figure 1C**). Lastly, significantly lower mRNA levels were observed for *Dbp* after challenge with each of the PAMPs when compared to the controls (**Figure 1D**). Consistent with what has been previously shown, out of all the clock associated genes examined, *Dbp* expression tends to be most affected by PAMP challenge²³.

FIGURE LEGENDS:

Figure 1: Altered clock gene expression in mouse splenocytes after *ex vivo* PAMP challenge. Splenocytes were isolated at ZT13 and challenged with LPS, ODN1826, or HKLM. Relative clock mRNA levels (normalized to β -actin) were determined by qPCR 3 h after challenge. Each data point represents expression level for 1 animal. Experimental mean + SEM are given. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The indicated challenges were significantly different from the control (unchallenged cells) as per one-way ANOVA with the Dunnett's post hoc test.

DISCUSSION:

Within this protocol, a microvolume spectrophotometer can be used to quantify and assess the purity of the RNA being used in determining gene expression. Nucleic acids absorb UV light at 260 nm, proteins typically absorb light at 280 nm, while other potential contaminants used during an RNA extraction procedure (e.g., phenol) are detectable at 230 nm. Therefore, by assessing the

absorbance (A) ratio at 260/280 nm (RNA to protein) and 260/230 nm (RNA to non-protein contaminants) the quality of the RNA can be assessed. High quality RNA has an A260/280 ratio between 1.8 – 2.1, as lower ratios indicate protein contamination. A pure RNA sample will have an A260/230 ratio of 2.0.

When determining the relative expression of a target gene (*i.e.*, *Per2*, *Clock*, *Rev-erba*, and *Dbp*), an endogenous control gene (a gene in which expression levels do not differ between samples) must also be selected. Relative expression of the target gene is then normalized to the expression of the endogenous control gene. Differences in starting material (number of splenocytes), variation in reverse-transcriptase efficiency, varying rates of RNA degradation, *etc.*, will be corrected for by the endogenous control gene (*β -actin* in this protocol). However, it is wise to verify that the treatment being examined does not alter expression of the endogenous control gene. This can be accomplished by assessing *β -actin* levels from several replicates of an equal number of cells (treatment vs. non-treatment). In theory, their *β -actin* levels should be identical. Another approach to guard against endogenous control variation would be to use a panel of endogenous controls (*e.g.*, *β -actin*, *Gapdh*, and 18S rRNA gene).

When examining the impact of PAMPs on the molecular clock, the time of day when mice are euthanized and splenocytes are subsequently challenged must be taken into consideration. *Tlr* expression and responsiveness has previously been shown to demonstrate time-of-day dependent variation^{8,15}, therefore, a time of day when TLR responsiveness is at its peak, could result in a greater influence on the clock. Furthermore, expression of molecular clock genes will also fluctuate throughout the day in splenocytes, therefore, a reduction of clock gene expression due to PAMP challenged would be most significant if examined during the time of peak expression⁹. Since *Dbp* and *Rev-erba* have been shown to demonstrate significant expression peaks in splenocytes and splenic immune cells around the light-dark interphase^{8,9,23,24} (ZT12), in the current method, cells were isolated and challenged at ZT13 in order to have a greater chance at detecting a reduction in these genes. Conversely, a PAMP that could increase clock expression, would most likely be observed if looking at a time of day when clock expression is at its lowest.

Since mice are nocturnal animals, their rest phase is during the light period, which corresponds to human activity. Therefore, ideally, mice will be housed in a room with minimal traffic and one that contains a white-noise machine in order to reduce daytime disturbances as this could disrupt the circadian rhythms of the mice. Furthermore, cage changes should be done well in advance of the experiment date. Any work in the animal room (including euthanasia of the animals) during the dark period, should be conducted under red light in order to avoid disrupting the rhythms of the mice.

Diurnal rhythms are subjected to environmental stimuli (*e.g.*, light or food), which are termed zeitgebers. In the case of a 12-h light / 12-h dark cycle, the zeitgeber (*i.e.*, light) resets the clock to a 24-h period. While most diurnal rhythms are circadian (*i.e.*, daily rhythms that occur in the absence of an external cue), they are not true circadian rhythms until they have been shown to oscillate with an approximate 24-h period under constant environmental conditions. Therefore,

this procedure could be performed using mice under constant conditions, which would entail entraining mice to the light-dark cycle as described above, but then holding the animals in constant darkness for 3 days prior to sampling. This type of experiment is referred to as a dark-dark (DD) experiment and the time point of sampling would be referred to as CT (circadian time), not ZT.

While this method can identify PAMPs that alter clock gene expression within splenocytes, it does not take into account how these PAMPs affect the master clock or peripheral clocks throughout the body. Since the spleen is composed of a heterogeneous population of cells, individual PAMPs could impact each cell type differently. For example, *Tlr9* expression rhythms in the mouse spleen differ between splenocytes, macrophages, B cells, and DCs¹⁵. Additionally, *Tlr1*, *Tlr3*, *Tlr4*, *Tlr6*, *Tlr7*, and *Tlr8* displayed significant daily oscillations in an adherent splenocyte population but only *Tlr2* and *Tlr6* experience daily oscillations in enriched splenic macrophages²⁴. Therefore, in order to investigate the outcome of a challenge on individual cell types, cells could be isolated via magnetic cell sorting, as previously described^{9,15} and then subsequently challenged. Additionally, the splenic cell population fluctuates over the daily cycle, which could also play a role in sensitivity to a particular PAMP and subsequent impact on the clock⁸.

This method allows for the isolation of a large number of immune cells that consist predominately of B cells (~58%), T cells (~21%), dendritic cells (~5%), and macrophages (~4%)²⁵. The large number of cells provides the opportunity to challenge splenocytes with a variety of PAMPs in a single experiment. The splenocyte isolation procedure is very easy to perform, can be completed within minutes (depending on the number of animals), and with minimal animal or cellular manipulation, which is essential when examining the molecular clock because as mentioned above, these actions can disrupt the timing of the clock as well as clock-controlled genes. The results for this procedure were highly reproducible, as significance between challenged and unchallenged cells was achieved with just three animals and the results were consistent with previously published work²³ (**Figure 1**). It should be noted that increasing the number of animals per group might have revealed statistically significant differences between a challenge group and control (*e.g.*, ODN 1826 and *Rev-erb α*).

Moving forward, while this protocol only addresses the acute effects on clock gene expression after PAMP challenge, it could provide proof of principle for further investigation. For example, this assay could be used as a model to decipher the molecular mechanisms regarding TLR – PAMP interaction and how it influences the molecular clock. It could also be used to determine the length of time it takes for the molecular clock to recover after a PAMP challenge, which could be determined by conducting a time-course experiment (*i.e.*, assessing expression after varying times post challenge). As mentioned above, subsequent experiments could be performed to examine PAMP challenge on specific splenocyte cell populations. Since several pathogens stimulate multiple TLRs upon infection, it would be interesting to use this protocol to investigate if challenging with multiple PAMPs have a synergistic effect on clock gene expression.

ACKNOWLEDGMENTS:

This work was supported by Faculty Research grants from the College of Arts and Sciences Dean's Office at the University of Hartford.

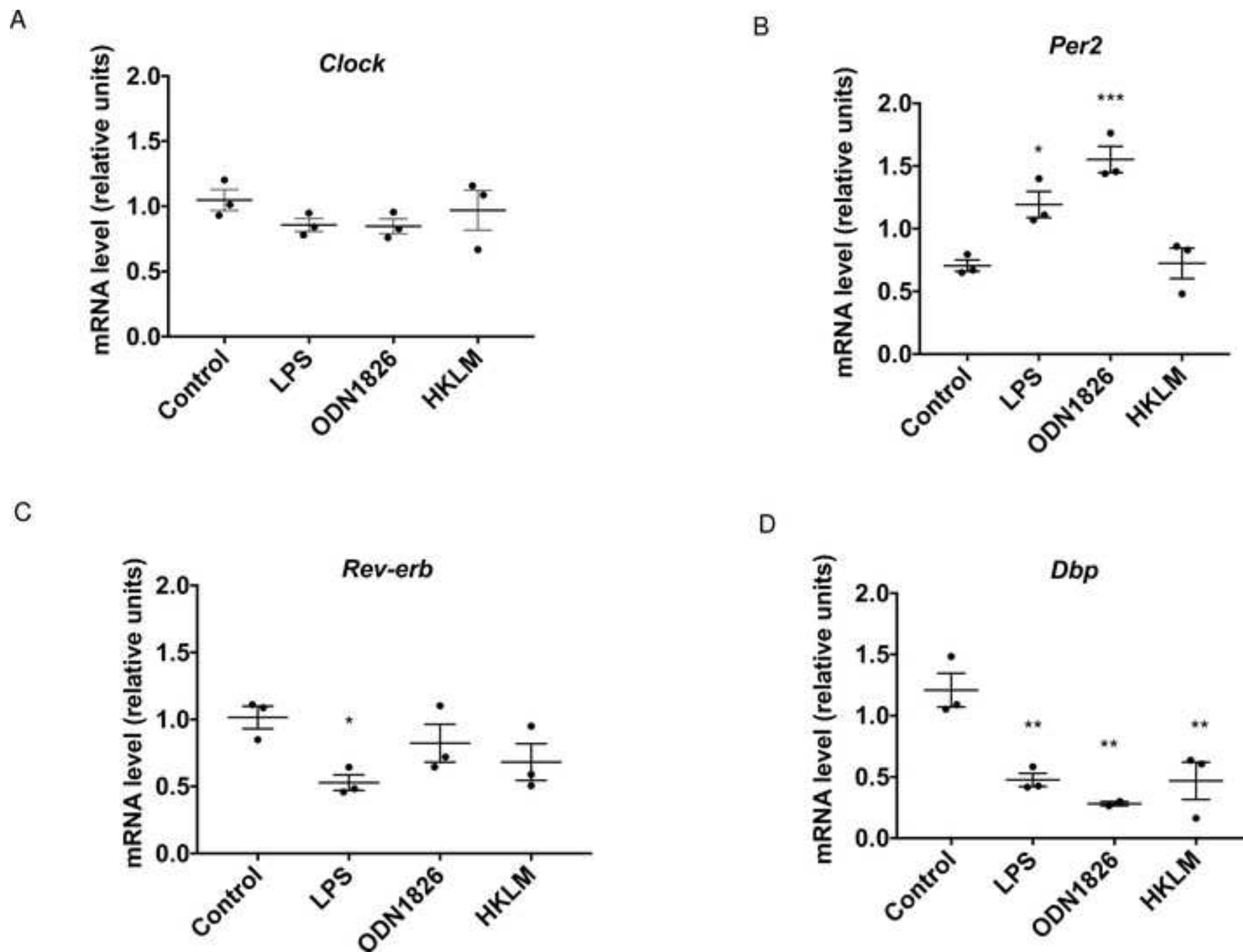
DISCLOSURES:

The author has nothing to disclose.

REFERENCES:

1. Bell-Pedersen, D. *et al.* Circadian rhythms from multiple oscillators: lessons from diverse organisms. *Nat. Rev. Genet.* **6**, 544–56 (2005).
2. Mohawk, J. A., Green, C. B. & Takahashi, J. S. Central and Peripheral Circadian Clocks in Mammals. *Annu. Rev. Neurosci.* **35**, 445–462 (2012).
3. Balsalobre, A., Damiola, F. & Schibler, U. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* **93**, 929–937 (1998).
4. Yoo, S.-H. *et al.* PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 5339–46 (2004).
5. Yamazaki, S. Resetting Central and Peripheral Circadian Oscillators in Transgenic Rats. *Science*. **288**, 682–685 (2000).
6. Lowrey, P. L. & Takahashi, J. S. Genetics of circadian rhythms in mammalian model organisms. *Adv. Genet.* **74**, 175–230 (2011).
7. Curtis, A. M., Bellet, M. M., Sassone-Corsi, P. & O'Neill, L. A. J. Circadian Clock Proteins and Immunity. *Immunity* **40**, 178–186 (2014).
8. Keller, M. *et al.* A circadian clock in macrophages controls inflammatory immune responses. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 21407–12 (2009).
9. Silver, A. C., Arjona, A., Hughes, M. E., Nitabach, M. N. & Fikrig, E. Circadian expression of clock genes in mouse macrophages, dendritic cells, and B cells. *Brain. Behav. Immun.* **26**, 407–413 (2012).
10. Kawai, T. & Akira, S. review The role of pattern-recognition receptors in innate immunity : update on Toll-like receptors. *Nat. Publ. Gr.* **11**, 373–384 (2010).
11. Marpegán, L., Bekinschtein, T. A., Costas, M. A. & Golombek, D. A. Circadian responses to endotoxin treatment in mice. *J. Neuroimmunol.* **160**, 102–109 (2005).
12. Okada, K. *et al.* Injection of LPS Causes Transient Suppression of Biological Clock Genes in Rats. *J. Surg. Res.* **145**, 5–12 (2008).
13. Haimovich, B. *et al.* In vivo endotoxin synchronizes and suppresses clock gene expression in human peripheral blood leukocytes. *Crit. Care Med.* **38**, 751–758 (2010).
14. Curtis, A. M. *et al.* Circadian control of innate immunity in macrophages by miR-155 targeting Bmal1. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 7231–6 (2015).
15. Silver, A. C., Arjona, A., Walker, W. E. & Fikrig, E. The Circadian Clock Controls Toll-like Receptor 9-Mediated Innate and Adaptive Immunity. *Immunity* (2012). doi:10.1016/j.immuni.2011.12.017
16. Gibbs, J. E. *et al.* The nuclear receptor REV-ERB α mediates circadian regulation of innate immunity through selective regulation of inflammatory cytokines. *PNAS* **109**, 582–587 (2012).
17. Zee, P. C., Attarian, H. & Videnovic, A. Circadian rhythm abnormalities. *Contin. Lifelong Learn. Neurol.* **19**, 132–147 (2013).

18. Bovbjerg, D. H. Circadian disruption and cancer: sleep and immune regulation. *Brain. Behav. Immun.* **17**, S48–S50 (2003).
19. Fu, L. & Lee, C. C. The circadian clock: pacemaker and tumour suppressor. *Nat. Rev. Cancer* **3**, 350–361 (2003).
20. Germain, A. & Kupfer, D. J. CIRCADIAN RHYTHM DISTURBANCES IN DEPRESSION. *Hum. Psychopharmacol.* **23**, 571–585 (2008).
21. JoVE Science Education Database. Basic Mouse Care and Maintenance. JoVE, Cambridge, MA (2018).
22. Leary, S. *et al.* AVMA Guidelines for the Euthanasia of Animals : 2013 Edition. (2013).
23. Silver, A. C. Pathogen-associated molecular patterns alter molecular clock gene expression in mouse splenocytes. *PLoS One* 12–15 (2017). doi: 10.1371/journal.pone.0189949
24. Silver, A. C. *et al.* Daily oscillations in expression and responsiveness of Toll-like receptors in splenic immune cells. *Heliyon* e00579 (2018). doi:10.1016/j.heliyon.2018.e00579
25. Jackson Lab. Flow-cytometric Anal. 11 strains mice. MPDJaxpheno6. Mouse Phenome Database web Resour. (RRIDSCR_003212), Jackson Lab. Bar Harb. Maine USA. <https://phenome.jax.org>



Name of Material/ Equipment	Company	Catalog Number
Frosted slides	Fisher	12-550-343
Cell strainers	Fisher	22363547
Lipopolysaccharide	InvivoGen	ltrl-eklps
ODN1826	InvivoGen	Tlrl-1826-1
HKLM	InvivoGen	Tlrl-hklm
RPMI 1640	Gibco	11875-093
PBS	Gibco	20012-043
RNeasy Mini Kit	Qiagen	74104 or 74106
RNase-Free DNase Set	Qiagen	79254
6-well cell culture plate	Denville	T1006
50 ml tubes	Corning	352070
15 ml tubes	Corning	352097
High Capacity cDNA Reverse Transcription Kit	ThermoFisher	4368814
TaqMan Gene Expression Assays b-actin	ThermoFisher	Mm00607939_s1
TaqMan Gene Expression Assays Per2	ThermoFisher	Mm00478113_m1
TaqMan Gene Expression Assays Rev-erba	ThermoFisher	Mm00520708_m1
TaqMan Gene Expression Assays Bmal1	ThermoFisher	Mm00500226_m1
TaqMan Gene Expression Assays Dbp	ThermoFisher	Mm00497539_m1
qPCR machine StepOnePlus	ThermoFisher	
TaqMan Gene Expression Master Mix	ThermoFisher	4369016
MicroAmp Fast 96-well reaction plate (0.1 ml)	ThermoFisher	4346907
Statistical Analysis Software	Prism 7.0a	



1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

Mouse splenocytes used to assess pathogen-associated molecular
pattern influence on molecular clock expression

Author(s):

Adam C. Silver

Item 1 (check one box): The Author elects to have the Materials be made available (as described at

<http://www.jove.com/author>) via: ☒ Standard Access ☐ Open Access

Item 2 (check one box):



The Author is NOT a United States government employee.



The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.



The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: **"Agreement"** means this Article and Video License Agreement; **"Article"** means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; **"Author"** means the author who is a signatory to this Agreement; **"Collective Work"** means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; **"CRC License"** means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; **"Derivative Work"** means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; **"Institution"** means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; **"JoVE"** means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments*; **"Materials"** means the Article and / or the Video; **"Parties"** means the Author and JoVE; **"Video"** means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such

statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's


expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name: Adam C. Silver
Department: Biology
Institution: University of Hartford
Article Title: Mouse Splenocytes used to Assess Pathogen-Associated Molecular Pattern
Glock Expression
Signature:  Date: 2/15/18

Please submit a signed and dated copy of this license by one of the following three methods:

- 1) Upload a scanned copy of the document as a pdf on the JoVE submission site;
- 2) Fax the document to +1.866.381.2236;
- 3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

>>This was completed.

3. Please define all abbreviations before use.

>>This was completed.

4. Please use SI units, e.g. please use “ μL ” instead of “ μl ”, “mL” instead of “ml”. Please leave a white space between the values and the units.

>>The changes were made.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (TM), registered symbols ([®]), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: “TaqMan”, “StepOnePlus”, “NanoDrop”, etc.

>>Commercial language was removed.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

>>This was completed.

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. The Protocol steps should contain only 2-3 actions per step and a maximum of 4 sentences per step.

>>This was completed.

8. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

>>This was completed.

9. For steps that involve software or analyzing tools, please make sure to provide all the details such as “click this”, “select that”, “observe this”, etc. Please mention all the steps that are necessary to

execute the action item. Please provide details so a reader may replicate your analysis including buttons clicked, inputs, screenshots, etc. This is the level of detail we're looking for. Please keep in mind that software steps without a graphical user interface cannot be filmed.

>>This was completed.

10. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

>>An ethics statement was provided.

11. Please include the age, sex and strain of the animals.

>>>The information was added.

12. Protocol: 1.1: Please include all the conditions for keeping the animals or refer to appropriate references. Please define all abbreviations before use.

>>ZT was defined and a reference was inserted describing basic animal husbandry.

13. Protocol: 2.2: How much of each solution should be prepared? Please include the containers.

>>The information was added

14. Protocol: 2.3: How the culture media is warmed? Using what? How much PBS is needed? What is the pH of the buffer?

>>The information was added

15. Protocol: 2.4: Please include the pipette size.

>>The information was added

16. Protocol: 2.5: How much ethanol is needed?

>>The information was added

17. Protocol: 3.1: Please clearly describe the euthanasia process or refer to appropriate references. Please use the imperative tense for all the sentences in the protocol step. Please attention that euthanasia process can not be filmed, so please do not highlight this steps.

>>The highlight was removed. The following sentence was also removed, "For this particular experiment, splenocytes were isolated from mice that were sacrificed at ZT13" as the timing of sampling is described in the discussion. Details regarding the euthanasia process was added as was a reference.

18. Protocol: 3.2: How to cut away the fur? Using what?

>>This information was added.

19. Protocol: 3.3: Please use sub-steps for long protocol steps.

>>sub-steps were added

20. Protocol: 3.5: Please use the imperative tense for all the sentences in the protocol steps.

>>The step was reworded

21. Protocol: 3.7: How to transfer the medium?

>>This information was added

22. Protocol: 3.8: What is the temperature of the centrifugation?

>>This information was added.

23. Protocol: 4.1, 4.2: Please use the imperative tense for all the sentences in the protocol steps. Please avoid using any commercial language, please use a generic term instead.

>>The changes were made and a sub-step was added

24. Protocol: 4.3: The Protocol steps should contain only 2-3 actions per step and a maximum of 4 sentences per step. Please use sub-steps. Please clearly describe the actions in the imperative tense. Please include the pipette size and the containers.

>>The relevant information was included and the step was sectioned into sub-steps.

25. Protocol: 5.1, 5.2, 5.4: Please avoid using any commercial language (e.g., StepOnePlus, TaqMan, etc.), please use a generic term instead.

>>These terms were removed

26. Protocol: 6.1: Please use the imperative tense for all the sentences in the protocol steps. Please clearly describe the actions or refer to appropriate references.

>>The section was re-written

27. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file.

>>The table was removed

28. If you are reusing figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [AUTHOR] et al.[REFERENCE]".

>>This does not apply

29. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please list all the materials, equipment, instrument, and software used in your work.

>>The table has been modified

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

very nice summary of the relevance of the clock and what this technique will provide. Very detailed protocol and nice discussion of how to interpret the data

Major Concerns:

None

Minor Concerns:

None

Reviewer #2:

Manuscript Summary:

This paper describes a method to use mouse splenocytes to assess Pathogen-Associated Molecular Pattern Influence on clock gene expression. Over all, this paper is well written, and clearly stated.

Major Concerns:

1. In this paper, the author collected splenocytes from the tissue, and wanted to isolation the immune cells from the splenocytes. But in this method, the author did not detect the cell type he/she got, so it is not sure whether the cell in this paper is immune cells or not.

>>While I did not demonstrate the splenic cell composition in this method, the immune cell percentages present in the spleen have been previously determined and confirmed by my colleagues

and I (unpublished data). The cell percentages are listed in the discussion. We used this splenocyte isolation protocol previously and subsequently worked with an adhere cell population:
<https://www.heliyon.com/article/e00579>

2. In the paper, after separated the cell from the splenocytes, the author used RPMI 1640 with 10% FBS to incubate the cell. As we know, FBS can induce circadian gene expression in mammalian tissue culture cells (PMID:9635423), and the cultured cells. So, the dissecting time does not have much meaning.

>>I have previously tried to trigger the clock *ex vivo* in splenocytes using the above method. However, I was unable to do so (unpublished data) and that was using 50% serum as was used in the paper (Balsalobre, 1998) mentioned by reviewer #2. If a serum shock using 50% serum did not reset the clock in these cells, then I do not believe 10% would.
>>More importantly, I have assessed expression of *Per2* and *Rev-erba* in splenocytes isolated over a 24-h period using the same method I am describing in this protocol. Daily changes in gene expression were observed (<http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0189949#sec013>), which demonstrates that the serum in the RPMI is not resetting the clock. If the clock was reset, expression would not change over the 24-h period.

Minor Concerns:

1. Line 44, it should make clear that the definition of the "master clock" in mammals or mice.

>>This information was added.

2. Line 82, "Animal Ethics Statement" should be stated.

>>An ethics statement was added

3. Line 82, Light intensity should give. Mouse age and gender should be made clear.

>>This information was added

4. Line 101, How long the time need for sterilize should give.

>>Scissors and forceps were originally autoclaved. The ethanol was used to prevent contamination and keep them 'clean.' This information was added to the protocol.

5. Line 105, "600 ml per sample", it is not clear the sample size, sample weight or cell number should give.

>>This information was added.

6. Line 157, it is better to give the RNA weight, not the volume.

>>In Protocol: 4.2 I added that up to 2 μg can be used. Since every well contains 10^6 cells, the amount of RNA that is extracted is very consistent and yields under 2 μg , therefore, I use 10 μl of RNA during the cDNA synthesis step.

7. Line 181 and 187, again, cDNA weight should also give.

>>In Protocol: 5.2, I added the range of cDNA that can be used for the reaction.

8. Figure 1, the SD or SEM should be given in the figure, not only the mean value.

>>The SEMs were added to the figure.

Reviewer #3:

In this manuscript, Dr. Silver describes a protocol to measure the impact of PAMPs on clock gene expression in splenocytes. The protocol consists in harvesting spleens from mice, culturing the splenocytes in the presence or absence of LPS or other PAMPs, preparing RNA and using it for RT-qPCR for clock genes. Although the manuscript is generally clear and well written, and the protocol might be useful to some readers/viewers, it would benefit from the clarifications requested below.

Main concerns:

1) This protocol is extremely basic. Culture of primary splenocytes is routine use in numerous labs, as qPCR is too. I know that JoVE is fine with publishing even very basic approaches, so I guess that publishing this manuscript is relevant. I have not checked previous JoVE manuscripts/videos, but I would have assumed that there are already some that describe either splenocyte culture or qPCR, perhaps both together. The novelty here is to present a protocol that brings the two together, in the context of clock gene expression. In any case, this protocol and video might be of interest to JoVE readers, but I feel that the author should better explain the novelty of what is presented.

>>While addressing the editor's and reviewers' comments, I believe this was accomplished.

2) The manuscript (and presumably the video too) should better explain why it is important to study the effect of PAMPs and TLR activation on clock genes. Such an explanation is absent both from the introduction and the discussion. A sentence on line 77 says "since disruptions of circadian rhythms can lead to serious pathology" as a rationale for the proposed approach. However, the protocol only addresses the acute effects of PAMPs, not the (longer term) effect on clock function itself.

>>I believe the following will better explain the usefulness of this protocol.

>>The following sentence was added to the introduction: "The molecular clock has been shown to modulate various aspects of the immune response, therefore, disruption of the molecular clock would most likely impair the proper time-dependent variation of the immune response."

>>>The following paragraph was added to the discussion: Moving forward, while this protocol only addresses the acute effects on clock gene expression after PAMP challenge, it could provide proof of principle for further investigation. For example, this assay could be used as a model to decipher the

molecular mechanisms regarding TLR – PAMP interaction and how it influences the molecular clock. It could also be used to determine the length of time it takes for the molecular clock to recover after a PAMP challenge, which could be determined by conducting a time-course experiment (*i.e.*, assessing expression after varying times post challenge). As mentioned above, subsequent experiments could be performed to examine PAMP challenge on specific splenocyte cell populations. Since several pathogens stimulate multiple TLRs upon infection, it would be interesting to use this protocol to investigate if challenging with multiple PAMPs have a synergistic effect on clock gene expression.”

3) A general comment about the Protocol section of the manuscript is that although well written, I feel that it should be more detailed, and should describe which aspects are critical for it to work, including tips to optimize the procedure. Since this is a very basic protocol, it will be read (and watched) by people who presumably know little about splenocyte culture, qPCR or clock genes. Hence, no details should be omitted and no basic knowledge assumed as known by the readers.

>>While addressing the editor’s and reviewers’ comments, I believe this was accomplished.

4) The section on animals should be detailed. How are they entrained in the LD conditions, under which conditions exactly?

>>More details were provided.

It should also be explained that the procedure could be done under constant darkness if one would be interested in splenocytes under circadian free-running conditions.

>>The following was added to the discussion: “Diurnal rhythms are subjected to environmental stimuli (*e.g.*, light or food), which are termed zeitgebers. In the case of a 12-hr light / 12-hr dark cycle, the zeitgeber (*i.e.*, light) resets the clock to a 24-hr period. While most diurnal rhythms are circadian (*i.e.*, daily rhythms that occur in the absence of an external cue), they not true circadian rhythms until they have been shown to oscillate with an approximate 24-h period under constant environmental conditions. Therefore, this procedure could be performed using mice under constant conditions, which would entail entraining mice to the light-dark cycle as described above, but then the animals would be held in constant darkness for 3 days prior to sampling. This type of experiment is referred to as a dark-dark (DD) experiment and the time point of sampling would be referred to as CT (circadian time), not ZT.”

Even more important, the reason for the time of collection should be explained. Why harvesting the spleens at ZT13? This is not a time of day (evening) where most people would normally do their procedures, so explanations are needed.

>>The following was added to the first paragraph of the discussion: “Since *Dbp* and *Rev-erb α* have been shown to demonstrate significant expression peaks in splenocytes and splenic immune cells around the light-dark interphase (ZT12), in the current method, cells were isolated and challenged at ZT13 in order to have a greater chance at detecting a reduction in these genes.”

Actually, based on what is already known in the literature about the circadian control of response to PAMPs, couldn't one expect that the response be different if spleens/splenocytes are taken at different times over the 24 h cycle? This would be important to mention, as a possible factor to consider (and test) in such experiments. (Or maybe this is not relevant, if the medium treatment resets the clock? See point 5, below.)

>>Yes, and this was addressed in the discussion: “When examining the impact of PAMPs on the molecular clock, the time of day when mice are sacrificed and splenocytes are subsequently challenged must be taken into consideration. *Tlr* expression and responsiveness has previously been shown to demonstrate time-of-day dependent variation^{9,23}, therefore, a time of day when TLR responsiveness is at its peak, could result in a greater influence on the clock. Furthermore, expression of molecular clock genes will also fluctuate throughout the day in splenocytes, therefore, a reduction of clock gene expression due to PAMP challenged would be most significant if examined during the time of peak expression¹⁰. Conversely, a PAMP that could increase clock expression, would most likely be observed if looking at a time of day when clock expression is at its lowest.”

5) I feel that the culture of the splenocytes and their stimulation is problematic when one considers that these cells have clocks which can be reset by serum treatment (like the one taking place when 3 ml of medium is added, line 134). Could this resetting by the serum mask the effect of PAMPs? For example, if a gene transcript is at high level in the cells, maybe one would not see a stimulation by a PAMP, but such an effect would be seen upon treatment at another clock time (time after synchronization of the cellular clocks). So maybe the protocol should include a wait of a few days after putting the splenocytes in culture (to let the clocks desynchronize) or conversely, take advantage of this clock synchronization and stimulate with PAMPs at different times after synchronization?

>>I have previously tried to trigger the clock *ex vivo* in splenocytes using the above method. However, I was unable to do so (unpublished data) and that was using 50% serum as was used in the paper (Balsalobre, 1998). If a serum shock using 50% serum did not reset the clock in these cells, then I do not believe 10% would.

>>More importantly, I have assessed expression of *Per2* and *Rev-erba* in splenocytes isolated over a 24-h period using the same method I am describing in this protocol. Daily changes in gene expression were observed (<http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0189949#sec013>), which demonstrates that the serum in the RPMI is not resetting the clock. If the clock was reset, expression would not change over the 24-h period.

6) Why harvest cells 3 h after PAMP stimulation (line 136)? Is the time course the same for all PAMPs? And for all clock (or other target) genes? A time course (e.g. 1-2-3-6-12h) would be more advisable.

>>While a 3 h timepoint was chosen for this protocol, a time course experiment would be very interesting as it would allow one to determine the length of time it takes the clock to recover from PAMP challenge. I added this potential experiment to the discussion.

7) Why isn't the qPCR part planned to be in the video? It is not less complex nor less tricky than splenocyte preparation and culture (in fact, it is probably more, for someone not experienced in qPCR).

>>I would leave that decision up to the editor. From what I have seen, protocols in JoVE frequently have links to previously established protocols. I assumed this protocol would have a link to qPCR set up. However, I would be fine with including it in the video.

8) A problem with this protocol is the complex cell population that is studied, which limits the interpretation of the data. This is somewhat addressed in the Discussion, but it should be expanded, for example to explain how to interpret the data despite this limitation (e.g. based on the known expression of the TLRs in the spleen cells?) and how it could be solved experimentally (e.g. purification of the cell subsets prior to stimulation). I feel that this limitation is more problematic than the advantages listed for the protocol (large cell numbers, little tissue manipulation).

>>The following was added to the discussion: "For example, *Tlr9* expression rhythms in the mouse spleen differ between splenocytes, macrophages, B cells, and DCs¹⁶. Additionally, *Tlr1*, *Tlr3*, *Tlr4*, *Tlr6*, *Tlr7*, and *Tlr8* displayed significant daily oscillations in an adherent splenocyte population but only *Tlr2* and *Tlr6* experience daily oscillations in enriched splenic macrophages²⁵. Therefore, in order to investigate the outcome of a challenge on individual cell types, cells could be isolated via magnetic cell sorting, as previously described^{10,16} and then subsequently challenged."

>>Additionally, this protocol can be used for proof of principle for more expanded studies. This is stated in the revised concluding paragraph in the discussion.

Actually, the list of cells (line 259) is not correct: among the splenocytes obtained by the technique as described, there would be no DCs or macrophages, which would require collagenase treatment to be released from the tissue; this needs to be corrected. Related to this is the point made by the author about the circadian rhythm of these cell types within the spleen: what could be the solution for this? Discuss.

>>I have previously used this isolation procedure and was able to obtain both macrophages/monocytes and dendritic cells: <https://doi.org/10.1016/j.heliyon.2018.e00579>

>>Keller et al. use a similar approach as to what I describe (i.e., no collagenase treatment) to isolate CD11b+ cells.

>>Also listed below is a reference for a protocol comparing DC isolation via cell strainer vs. collagenase treatment.

>>While DC and macrophage numbers are lower without collagenase, it is incorrect to say that DCs and macrophages would not be present.

A circadian clock in macrophages controls inflammatory immune responses

Maren Keller, Jeannine Mazuch, Ute Abraham, Gina D. Eom, Erik D. Herzog, Hans-Dieter Volk, Achim Kramer, Bert Maier

Proceedings of the National Academy of Sciences Dec 2009, 106 (50) 21407-21412; DOI: 10.1073/pnas.0906361106

Isolation of Mouse Spleen Dendritic Cells Andrew J. Stagg, Fiona Burke, Suzanne Hill, and Stella C.

Knight From: Methods in Molecular Medicine, vol. 64: Dendritic Cell Protocols Edited by: S. P. Robinson and A. J. Stagg © 2001 Humana Press Inc., Totowa, NJ

More specific comments:

- Line 3-4: The title should be revised to: "The use of mouse splenocytes to assess pathogen-associated molecular pattern influence on clock gene expression". (first few words revised to read better; and "molecular clock expression" — somewhat meaningless — revised to "clock gene expression")

>>The change was made

- Line 54: "Functional molecular clocks have been described..." In 2 of the 3 references cited here (Keller et al being the exception), only clock gene expression has been described. This is not sufficient to conclude that a "functional clock" is present in those cells. Assays to assess the cell-autonomous rhythmic gene expression or circadian function must be performed (e.g. rhythms in ex vivo culture) to conclude that there is a functional clock. Revise the sentence or change the references.

>>I removed one of the references. However, the Silver et al reference assessed clock gene expression under constant conditions in ex vivo splenic immune cells, which is what the reviewer is requesting (i.e., "rhythmic gene expression in ex vivo culture"). Therefore, I am confused as to why this reference would not be appropriate. The authors of this paper state, "Our data demonstrate that macrophages, DCs, and B cells enriched from mouse spleen possess functional molecular clocks as demonstrated by the daily oscillations in clock gene expression."

- Line 86: Define ZT at first occurrence.

>>The change was made.

- Lines, 97, 99, 118, and other occurrences: singular is "medium", not "media".

>>The changes were made.

- Line 105 (and 145): What is RLT buffer?

>>The following was added to the first place the buffer was used: "... Buffer RLT (a proprietary component of the RNA extraction kit that supports the binding of RNA to the silica membrane)..."

- Line 115: Cut only the fur, not also the tissue under it? And "left side of the mouse" is unclear: from which standpoint?

>>The following was added: "Using forceps, grab the peritoneum and carefully make an incision as not to damage the spleen."

>>"left side of the mouse" was changed to, "mouse's left side"

- Line 122: "between two sterile frosted slides": Mention that the tissue is grinded between the frosted parts. This is unclear. Also mention that the tissue and cells should remain wet and in the buffer at all times.

>>This information was added.

- Line 150: Briefly describe those manufacturer's instructions? Are there aspects of this manufacturer's protocol that are particularly tricky or that should be performed in a certain way?

>>While initially writing this protocol I asked the science editor about this issue. She told me that writing "according to manufacturer's instructions" would suffice.

- Line 154-155: Explain Nanodrop quantification and what the optimal range is. Also explain how to assess the purity of the RNA.

>>The following was added to the discussion: Within this protocol, a microvolume spectrophotometer can be used to quantify and assess the purity of the RNA being used in determining gene expression. Nucleic acids absorb UV light at 260 nm, proteins typically absorb light at 280 nm, while other potential contaminants used during an RNA extraction procedure (e.g. phenol) are detected at 230 nm. Therefore, by assessing the absorbance (A) ratio at 260/280 nm (RNA to protein) and A260/230 (RNA to non-protein contaminants) the quality of the RNA can be assessed. High quality RNA has an A260/280 ratio between 1.8 – 2.1, as lower ratios indicate protein contamination. A pure RNA sample will have an A260/230 ratio of 2.0.

>>The range of RNA used in the cDNA synthesis was added to the protocol.

- Line 191: Why use actin as the control? Explain that any gene used as control in the qPCRs must be verified to ensure that it does not vary with the treatment studied. Explain how.

>>The following paragraph was added: "When determining the relative expression of a target gene (*i.e.*, *Per2*, *Clock*, *Rev-erba*, and *Dbp*), an endogenous control gene (a gene in which expression levels do not differ

between samples) must also be selected. Relative expression of the target gene is then normalized to the expression of the endogenous control gene. Differences in starting material (number of splenocytes), variation in reverse-transcriptase efficiency, varying rates of RNA degradation, etc., will be corrected for by the endogenous control gene (*β-actin* in this protocol). However, it is wise to verify that the treatment being examined does not alter expression of the endogenous control gene. This can be accomplished by assessing *β-actin* levels from several replicates of an equal amount of cells (treatment vs. non-treatment). In theory, their *β-actin* levels should be identical. Another approach to guard against endogenous control variation would be to use a panel of endogenous controls (*e.g.*, *β-actin*, *Gapdh*, and 18S rRNA gene)."

- Lines 193-195: This data analysis point is very vague. A well validated approach such as the 2(-ddCt) must be used. Explain how. (See Livak KJ, Schmittgen TD. 2001. Methods 25:402-408.)

>>I have previously used this method:

Silver et al., The circadian clock controls toll-like receptor 9-mediated innate and adaptive immunity. *Immunity*, 2012

Silver et al., Circadian expression of clock genes in mouse macrophages, dendritic cells, and B cells. *Brain, Behavior, and Immunity*, 2012.

- Line 199: Use present tense as this is in the protocol ("is used").

>>This was changed.

- Line 263: Explain why it is essential to minimize animal and cell manipulations in clock studies.

>>The following was added: "because as mentioned above, these actions can disrupt the timing of the clock as well as clock controlled genes."

- Lines 264-265: A n=3 is low, and could be insufficient depending on the stimulus used or the gene tested. Actually, the data provided in this manuscript suggest that there might be an effect of ODN1826 and HKLM on *Reverba* that does not reach significance due to a lack of power. If true, these data would argue for using a higher group size.

>>The following was added to the discussion: "It should be noted that increasing the number of animals per group might have revealed statistically significant differences between a challenge group and control (*e.g.*, ODN 1826 and *Rev-erbα*)."

- Lines 266-268: The sentence starts by "Moving forward" but the rest is just to state the primary objective of this manuscript. The manuscript would gain from ending on a description of other possible applications of this protocol, beyond PAMPs, beyond clock genes, and perhaps with more conditions and time points.

>>I added a paragraph in the discussion that addresses these points.

- Line 262: Reference 20 is incomplete.

>>The reference was fixed.

Reviewer #4:

Manuscript Summary:

This work introduced an method to analysis the molecular clock genes expression in mouse splenocytes after challenged ex vivo with the pathogen-associated molecular patterns (PAMPs), lipopolysaccharide (LPS), ODN1826, and heat-killed *Listeria monocytogenes*. Overall, from my impression, this work is well done indeed. It introduced detailed procedures about entrainment of animals, instruments and reagents preparation, mouse splenocyte isolation and challenge, RNA isolation and cDNA synthesis, Quantitative PCR, and Statistical analysis. People who are interested to investigate the pathogens influence on splenocyte molecular clock work can easily followed the protocols to achieve their research targets. Therefore, I recommend to publish this manuscript without any hesitate.

Major Concerns:

No

Minor Concerns:

No