EMBO reports

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# Parasite-induced ER stress response in hepatocytes facilitates Plasmodium liver stage infection

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**Review timeline:**

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| Editorial Decision: | 05 January 2015 |
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## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Nonia Pariente

1st Editorial Decision 05 January 2015

We have now received reports from the two referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although all the referees find the topic of interest and in principle suitable for publication in EMBO reports, they raise critical concerns regarding the conclusiveness of the findings due to possible off-target effects of tunicamycin, as well as some other issues that would need to be addressed before we can consider publication of your study.

Given that both provide constructive suggestions on how to strengthen the work, I would like to give you the opportunity to revise your manuscript. All concerns seem reasonable and should therefore be addressed. If the referee concerns can be adequately addressed, we will be happy to accept your manuscript for publication. Please note that we would require that a (preferably) genetic means of increasing ER stress in vivo in a specific manner be used, as referee 1 requests, rather than reverting to in vitro work -possibility mentioned by referee 2.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, please contact me if I can be of any assistance.

 

REFEREE REPORTS:

Referee #1:

In their manuscript, "A role for hepatic XPB1 and CREBH pathways during Plasmodium liver stage infection", Inacio and colleagues argue that ER stress is induced by the malaria liver stage parasite and that the XBP1 / CREBH pathways which are activated by this stress are important for optimal liver stage development. While this finding is interesting, the data are somewhat preliminary and a series of more robust controls need to be performed.
Major points:
1. Authors make major use of the compounds tunicamycin in order to induce ER stress. Although they show compelling data that this does, in fact, induce ER stress in their host cells and mice, they do not rule out the fact that tunicamycin is directly inducing ER stress - or other off target effects - in parasites directly. In fact, tunicamycin has been shown to affect Plasmodium blood stages. Although the effect on blood stages is to inhibit infection and the effect here is to boost infection, it is not clear that the compound is not directly interacting with the parasite-intrinsic ER-stress pathway. Furthermore, this compound has many effects, some of which have nothing to do with the ER-Stress pathway. This should be resolved with both the following experiments:
a. Authors should investigate the use of a transgenic mouse model which boosts levels of ER-Stress without the use of a small molecule that has potential off-target effects
b. The authors should ensure that tunicamycin has no effects on sporozoites by pre-incubating the sporozoites with the compound, and then monitoring infection/development of liver stages.
2. The authors show that liver stage infection is increased in response to ER stress (tunicamycin). However, it is not clear if this is an effect on invasion or development. Authors should perform experiments at different time points after infection (early invasion, 24h, etc.) to elucidate the point at which ER stress is important for liver stage infection. This should be a reasonably straight forward experiment to perform with their Luciferase expressing parasites.
3. On page 8, the authors describe an experiment where they compare liver stage burden in Xbp1 heterozygotes to knockout animals. Why did they use heterozygotes instead of wild-type mice in this case? It seems more appropriate to use wild-type animals since it is not clear what the level of Xbp1 is in heterozygote animals and thus the experiment is difficult to interpret.
4. Different species of Plasmodium parasites have different requirements of their host hepatocytes. Are their findings generalizable to Plasmodium liver stages which affect humans (i.e. Plasmodium falciparum)?
5. Did XBP1 and CREBH come up in either the transcriptomic or proteomic analyses? It is surprising that they are not mentioned, given how strongly they influenced infection in later experiments.
6. Authors should put their findings in the context of other pathogens (perhaps in the discussion section). For example, is the the ER-stress pathway a typical way in which intracellular pathogens evade host defenses or generate nutrients from their host or is their finding Plasmodium-specific?
Minor points:
7. Authors should put graphs in Fig. 2C on the same y-axis so the reader can easily compare.
8. Does Ad-CREBH affect hepatocyte viability? Does it induce liver damage? Authors could monitor ALT levels in mouse sera after Ad-CREBH administration to determine this.
9. In figure 4C, authors show down-regulation of the CREDB transcript after Ad transduction. Can this be shown on the protein level? It is hard to interpret the results without knowing the level of knockdown on the protein level in hepatocytes.

Referee #2:

In 2009 (Albuquerque et al, BMC Genomics) the same group showed a signature of stress response in Hepa 1-6 infected by Plasmodium berghei, including an early over-expression of ER-stress related genes as trib3, nupr1, ddit3 and atf4. The manuscript by Inacio et al is the continuation of this work and describes the interplay between Plasmodium berghei and ER stress responses during hepatoma/ hepatocyte infection. Transcriptional and proteomics analysis of infected hepatoma cell lines confirmed an over-expression of several markers associated to ER stress responses during

 

early-middle cell infection (6-24h). Using a high systemic dose of tunicamycin, an inhibitor of N-glycosylation and inducer of ER stress, the authors observed a 3 to 7-fold increase in the parasite load in the liver that matched a ~4-fold increase in the numbers of EEFs at 42h post-infection. Conditional depletion of XBP1 leaded to a ~2-fold decrease in parasite load and numbers in the liver. Similarly knockdown of CREBH leaded to a ~2-fold decrease in hepatocyte infection ex vivo and in vivo.

The participation of ER stress responses in the development of parasite inside hepatocytes is certainly an interesting and important concept in the field, but before accepting this manuscript I would like to clarify a few experimental points.

Major points:

1. In the experiment of figure 2, the authors used a high dose of tunicamycin (TM) (1mg/kg) which is known by its potent liver toxicity. Although this dose can be considered a sublethal dose, it is at the border of the lethal effects of TM (50% lethal dose, 2.0 mg/kg - please refer to the charter 3 of Morin & Bernacki, Cancer Res. 1983; 43(4):1669-74). Indeed, in figure 2E (panel TM, Hoechst staining) the nuclei of hepatocytes treated with TM are smaller and more condensed than in the control. Since TM inhibits N-glycosylation, causing ER stress and eventually cell death, it is hard to discriminate which effect of TM is responsible for the increase in the numbers of EEFs observed in TM-treated animals. Moreover TM will not target only hepatocytes. Other cells affected by TM could be responsible by this increase.
As the authors can determine the level of ER stress by the splicing of XBP1, a minimal dose of TM capable of exerting the ER stress without causing toxic effects could be determined and used. Conversely, the use of other well established inducers of ER stress could be used to rule out a possible effect of inhibition of N-glycosylation in the observed phenomenon.
Since the first observation of over-expression of markers of ER stress during infection were done using hepatoma cell lines and to avoid the systemic action of stress inducers in other cells which could play a role in the development of parasites, I understand that in vitro experiments using Hepa 1-6/primary hepatocytes could bring cleaner and more controlled results regarding the specific participation of hepatoma/hepatocyte ER stress responses in the parasite development.

2. What is the p-value of panel 3E?

3. In the figure 4 the authors showed a nice association between the knockdown of CREBH mRNA and parasite infection of primary hepatocytes ex vivo. I was quite surprised to see the same extent of decrease in vivo since the transduction of hepatocytes in vivo should be much less important than what was observed ex vivo. Did the authors quantify the levels of CREBH mRNA in hepatocytes after in vivo transduction like done in vitro? Is it possible that the knockdown of uninfected/ adjacent cells are responsible for the decrease in the infection?

Minor comments:

- The rational of using Hepa 1-2 and moving to Huh7 and vice-versa is not clear in figures 1 and 3, respectively.
- Page 8: "we observed that the effect in Plasmodium liver load is due to an alteration in numbers of EEFs rather then in their development. Our results show that Xbp1Δ livers exhibit a reduction in the number of infected cells in (Figure 3E), suggesting a direct effect of XBP1 regulated pathways on Plasmodium liver stage development.". This phrase is confusing because uses twice the word development with different meanings. Please rephrase it.

- Figure 4, Panel B. There is a problem in the normalization of values for the Ad-Us group, which average is not at 100%. The y-axis title seems to be wrong (primary hepatocytes not liver).
- Page 2: "In vivo UPR activation strongly increases Plasmodium exoerythrocytic form (EEF) numbers. " : In vivo tunicamycin... ?
- Page 2: "CREBH deletion": CREBH knockdown?
- Page 8: "rather then": rather than
- Page 11: "heat-activated Fetal Bovine Serum": inactivated?
- Page 12: reference is missing (Lee et al., 2008a).

 

1st Revision - authors' response 09 April 2015

Referee #1:

In their manuscript, "A role for hepatic XPB1 and CREBH pathways during Plasmodium liver stage infection", Inacio and colleagues argue that ER stress is induced by the malaria liver stage parasite and that the XBP1 / CREBH pathways which are activated by this stress are important for optimal liver stage development. While this finding is interesting, the data are somewhat preliminary and a series of more robust controls need to be performed.

***We want to thank the reviewer for the insightful comments, which we believe have helped us improve the quality of the manuscript. We have now addressed all the reviewer's concerns and suggestions and modified the manuscript accordingly.***

Major points:
1. Authors make major use of the compounds tunicamycin in order to induce ER stress. Although they show compelling data that this does, in fact, induce ER stress in their host cells and mice, they do not rule out the fact that tunicamycin is directly inducing ER stress - or other off target effects - in parasites directly. In fact, tunicamycin has been shown to affect Plasmodium blood stages. Although the effect on blood stages is to inhibit infection and the effect here is to boost infection, it is not clear that the compound is not directly interacting with the parasite-intrinsic ER-stress pathway. Furthermore, this compound has many effects, some of which have nothing to do with the ER-Stress pathway. This should be resolved with both the following experiments:

a. Authors should investigate the use of a transgenic mouse model, which boosts levels of ER-Stress without the use of a small molecule that has potential off-target effects.

***We agree with the reviewer that using a genetic model is always beneficial. Still, given that the ER stress response is not controlled by any specific gene, there is no transgenic mouse model that exhibits a constant induced ER stress response. Instead it is the presence of misfolded proteins inside the lumen of the ER that induce such a response. Extensive literature reports the induction of ER stress with chemical agents and among them tunicamycin is the most commonly used (Oslowski and Urano, 2011). We now show that the effect of tunicamycin on infection is dose-dependent and fully correlates with XBP1 splicing (Figure 2A). Importantly, the effect of TM in infection is corroborated by our data using XBP1-deficient mice in which one of the ER stress branches (XBP1-dependent) is impaired. Indeed, while tunicamycin treatment leads to an increase in infection, XBP1 deficiency causes a decrease in parasite liver load.***

b. The authors should ensure that tunicamycin has no effects on sporozoites by pre-incubating the sporozoites with the compound, and then monitoring infection/development of liver stages.

***As the reviewer mentioned, tunicamycin was reported to affect Plasmodium blood stages. We highlight, however, that this was achieved by incubation of infected erythrocytes for 96 h with tunicamycin (Udeinya and Van Dyke, 1981). Our experimental setup was completely distinct. ER stress was induced in mice by intraperitoneal injection of tunicamycin 8 h prior Plasmodium sporozoite infection. Still, we have now pre-incubated sporozoites with tunicamycin (10 µg/ml, commonly used in in vitro assays) and show that this treatment does not impact infection by sporozoites (now supplementary Figure 1C).***

2. The authors show that liver stage infection is increased in response to ER stress (tunicamycin). However, it is not clear if this is an effect on invasion or development.

 

Authors should perform experiments at different time points after infection (early invasion, 24h, etc.) to elucidate the point at which ER stress is important for liver stage infection. This should be a reasonably straight forward experiment to perform with their Luciferase expressing parasites.

***This experiment has been performed. The data clearly show that the effect of tunicamycin in infection is not in invasion or the early stages of infection, as 14 h after infection parasite liver load is similar in both control and tunicamycin-treated mice. The effect only becomes evident 24 h after infection (Figure 2F).***

3. On page 8, the authors describe an experiment where they compare liver stage burden in Xbp1 heterozygotes to knockout animals. Why did they use heterozygotes instead of wild-type mice in this case? It seems more appropriate to use wild-type animals since it is not clear what the level of Xbp1 is in heterozygote animals and thus the experiment is difficult to interpret.

***The use of littermate controls to measure Plasmodium liver load is critical as small differences in genetic background can strongly impact infection. We have used $Xbp1^{flox/flox;cre}$ and $Xbp1^{flox/+;cre}$ littermates due to colony constrains. Importantly and as expected, XBP1 protein expression´s levels is significantly different between the 2 genotypes (Figure 3C).***

4. Different species of Plasmodium parasites have different requirements of their host hepatocytes. Are their findings generalizable to Plasmodium liver stages which affect humans (i.e. Plasmodium falciparum)?

***All our data was obtained using the rodent malaria parasite P. berghei. Previous transcriptomic data from Mota and Kappe laboratories (Instituto de Medicina Molecular and Seattle Biomedical Research Institute, respectively) using P. berghei and P. yoelii show up-regulation of ER stress genes in both infections (Albuquerque et al., 2009). As such, we expect this to be a general aspect of Plasmodium liver stage infection. Still, we cannot assure that other Plasmodium spp cause the same effect on host cells and for that reason we have revised the text to make sure this is clear.***

5. Did XBP1 and CREBH come up in either the transcriptomic or proteomic analyses? It is surprising that they are not mentioned, given how strongly they influenced infection in later experiments.

***We could not detect CREBH neither in the transcriptomic or proteomic analysis. On the other hand XBP1 was detected in transcriptomic analysis (although did not pass the significance test) as well as its target gene Dnajb9. While a whole-cell proteomics analysis performed here is in agreement with a general ER stress response and UPR activation upon infection, a comprehensive ER proteome analysis requires doing ER fractionation, which was not the goal of the present study.***

6. Authors should put their findings in the context of other pathogens (perhaps in the discussion section). For example, is the the ER-stress pathway a typical way in which intracellular pathogens evade host defenses or generate nutrients from their host or is their finding Plasmodium-specific?

***We fully agree that this is an important point. The discussion does now include a paragraph addressing this pertinent comment.***

Minor points:

 

7. Authors should put graphs in Fig. 2C on the same y-axis so the reader can easily compare.

***The scale of Fig. 2C has been altered and includes now data from 6 different experiments.***

8. Does Ad-CREBH affect hepatocyte viability? Does it induce liver damage? Authors could monitor ALT levels in mouse sera after Ad-CREBH administration to determine this.

***No significant differences were observed between Ad-US (control) and Ad-CREBH transduced primary hepatocytes in terms of cell viability as measured by Alamar Blue (now Supplementary Figure 1D).***

9. In figure 4C, authors show down-regulation of the CREDB transcript after Ad transduction. Can this be shown on the protein level? It is hard to interpret the results without knowing the level of knockdown on the protein level in hepatocytes.

***We do now include a Western Blot showing a strong decrease in CREBH protein levels in livers transduced with Ad-CREBHi (now Figure 4F).***

Referee #2:

In 2009 (Albuquerque et al, BMC Genomics) the same group showed a signature of stress response in Hepa 1-6 infected by Plasmodium berghei, including an early over-expression of ER-stress related genes as trib3, nupr1, ddit3 and atf4. The manuscript by Inacio et al is the continuation of this work and describes the interplay between Plasmodium berghei and ER stress responses during hepatoma/ hepatocyte infection. Transcriptional and proteomics analysis of infected hepatoma cell lines confirmed an over-expression of several markers associated to ER stress responses during early-middle cell infection (6-24h). Using a high systemic dose of tunicamycin, an inhibitor of N-glycosylation and inducer of ER stress, the authors observed a 3 to 7-fold increase in the parasite load in the liver that matched a ~4-fold increase in the numbers of EEFs at 42h post-infection. Conditional depletion of XBP1 leaded to a ~2-fold decrease in parasite load and numbers in the liver. Similarly knockdown of CREBH leaded to a ~2-fold decrease in hepatocyte infection ex vivo and in vivo.

The participation of ER stress responses in the development of parasite inside hepatocytes is certainly an interesting and important concept in the field, but before accepting this manuscript I would like to clarify a few experimental points.

***We wish to thank this reviewer for the positive and incisive feedback. We have addressed now all the points raised, which has certainly contributed for a much more insightful story.***

Major points:

1. In the experiment of figure 2, the authors used a high dose of tunicamycin (TM) (1mg/kg) which is known by its potent liver toxicity. Although this dose can be considered a sublethal dose, it is at the border of the lethal effects of TM (50% lethal dose, 2.0 mg/kg - please refer to the charter 3 of Morin & Bernacki, Cancer Res. 1983; 43(4):1669-74). Indeed, in figure 2E (panel TM, Hoechst staining) the nuclei of hepatocytes treated with TM are smaller and more condensed than in the control. Since TM inhibits N-glycosylation, causing ER stress and eventually cell death, it is hard to discriminate which effect of TM is responsible for the increase in the numbers of EEFs observed in TM-treated animals. Moreover TM will not target only hepatocytes. Other cells affected by TM could be responsible by this increase.
As the authors can determine the level of ER stress by the splicing of XBP1, a minimal dose of TM capable of exerting the ER stress without causing toxic effects could be determined and used. Conversely, the use of other well established inducers of ER stress

 

could be used to rule out a possible effect of inhibition of N-glycosylation in the observed phenomenon.

***We have now tested different concentrations of tunicamycin and as the data clearly show the effect of tunicamycin on infection is dose-dependent and fully correlates with XBP1 splicing (Figure 2A). The minimal dose that impacts both XBP1 splicing and infection was 0.75 mg/Kg. Furthermore, we have performed a histopathological analysis of the livers of mice treated with the different concentrations and found no histomorphological differences between control and TM-treated mice (Supplementary Figure 1A).***

2. What is the p-value of panel 3E?

***It is *p<0.05 (p=0.015685). This has now been included in the figure.***

3. In the figure 4 the authors showed a nice association between the knockdown of CREBH mRNA and parasite infection of primary hepatocytes ex vivo. I was quite surprised to see the same extent of decrease in vivo since the transduction of hepatocytes in vivo should be much less important than what was observed ex vivo. Did the authors quantify the levels of CREBH mRNA in hepatocytes after in vivo transduction like done in vitro? Is it possible that the knockdown of uninfected/ adjacent cells are responsible for the decrease in the infection?

***We now show that efficiency of CREBH knockdown is similar in both in vivo and ex vivo transduction of Ad-CREBHi. We further show that this knockdown is also notorious at the protein level in vivo (Figure 4F). Given that CREBH is a hepatocyte-specific factor (Lee et al., 2011) we do not expect the participation of any other liver cells.***

Minor comments:

- The rational of using Hepa 1-2 and moving to Huh7 and vice-versa is not clear in figures 1 and 3, respectively.

***All in vitro data on this manuscript was obtained by using Hepa 1-6 hepatoma cells, with the exception of the proteomic analysis that was originally performed in the context of a different study. Still, the similarities between the microarray and proteomic data implying a general ER stress response and UPR activation upon infection prompted us to include these data as the basis for the rest of the study. Moreover, showing a similar response in two different cell lines reinforces the importance of the observation.***

- Page 8: "we observed that the effect in Plasmodium liver load is due to an alteration in numbers of EEFs rather then in their development. Our results show that Xbp1Δ livers exhibit a reduction in the number of infected cells in (Figure 3E), suggesting a direct effect of XBP1 regulated pathways on Plasmodium liver stage development.". This phrase is confusing because uses twice the word development with different meanings. Please rephrase it.

***This has now been altered in the text.***

- Figure 4, Panel B. There is a problem in the normalization of values for the Ad-Us group, which average is not at 100%. The y-axis title seems to be wrong (primary hepatocytes not liver).

***We apologize for this mistake. This has now been corrected.***

- Page 2: "In vivo UPR activation strongly increases Plasmodium exoerythrocytic form

 

(EEF) numbers. " : In vivo tunicamycin... ?
- Page 2: "CREBH deletion": CREBH knockdown?
- Page 8: "rather then": rather than
- Page 11: "heat-activated Fetal Bovine Serum": inactivated?
- Page 12: reference is missing (Lee et al., 2008a).

***All these aspects have been now corrected.***

### *References*

*Albuquerque SS, Carret C, Grosso AR, Tarun AS, Peng X, Kappe SH, Prudêncio M, Mota MM. Host cell transcriptional profiling during malaria liver stage infection reveals a coordinated and sequential set of biological events. BMC Genomics. 2009 Jun 17;10:270.*

*Lee JH, Giannikopoulos P, Duncan SA, Wang J, Johansen CT, Brown JD, Plutzky J, Hegele RA, Glimcher LH, Lee AH. The transcription factor cyclic AMP-responsive element-binding protein H regulates triglyceride metabolism. Nat Med. 2011 Jun 12;17(7):812-5.*

*Oslowski CM, Urano F. Measuring ER stress and the unfolded protein response using mammalian tissue culture system. Methods Enzymol. 2011;490:71-92.*

*Udeinya IJ, Van Dyke K. Concurrent inhibition by tunicamycin of glycosylation and parasitemia in malarial parasites (Plasmodium falciparum) cultured in human erythrocytes. Pharmacology. 1981;23(3):165-70.*

2nd Editorial Decision 11 May 2015

Thank you for your patience. We have now received the reports of the two referees that assessed the previous version of your study, both of whom are now supportive of publication (please see below). I have now also had time to go through your manuscript in detail in preparation for publication. I am thus writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

- As indicated in our previous correspondence, a citation to the publication in which the transcriptomics analysis shown in figure 1A was initially described should be included in the figure legend, as well as the database accession number to the data, if available.
In addition, we require that the proteomics analysis presented in figure 1C be deposited in a publicly available proteomics database and the accession number provided in the figure legend and in a data availability subsection at the end of the Materials & Methods.

- The identity of the error bars and number of samples analyzed needs to be included in the legends to figures 4 C and E, and Supp Fig 1C.

- Please include a brief discussion of the fact that both P. berghei and P. yoelii upregulate ER stress genes, thus suggesting that this is a general effect of Plasmodium infection. This raises the generality, and thus the impact, of the study.

- The format of the manuscript needs to be adapted to EMBO reports style. Although I will not insist on a specific text length, please merge the Results and Disussion section as per journal style. This should help shorten the text, which is currently longer than we normally accomodate. In addition, although basic Materials and Methods required for understanding the experiments performed must remain in the main text, additional detailed information may be included as supplementary material, which should be labeled Appendix.

 

After all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports. Do not hesitate to get in touch if I can be of any help in the preparation of your study for publication.

REFEREE REPORTS:

Referee #1:

the authors have responded adequately to all my comments and suggestions.

Referee #2:

Despite the fact that the authors chose not to test other ER stress agents in hepatocyte infection to strengthen the results obtained with tunicamycin, the authors answered all my questions and the manuscript has significantly improved after the modifications. Therefore, I don't see any major problem for publication.

2nd Revision - authors' response 01 June 2015

This is just a short message to let you know that I've submitted all the final requested files. Thank you so much for your patience.

3rd Editorial Decision 02 June 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication.

 