nature portfolio

Peer Review File

Leishmania major-induced alteration of host cellular and systemic copper homeostasis drives the fate of infection

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

By performing careful biochemical analyses in macrophage cell line infected with Leishmania major, the authors show that infection reduces the molecular mechanisms involved in copper transport towards the cell compartments where the parasites reside. First, they show colocalization of copper and Leishmania, as well as parasites in LAMP1+ phagolysosomes and the transporter ATPA7, which delivers Cu to stress parasites. Leishmania infection alters Cu physiology by inducing ATPA7 protein degradation, further facilitated by downregulation of chaperones and post-translational modifications, such as deglycosylation. In addition infection induces the endocytosis of CTR1 Cu importer and prevents transcription of CTR1. Following suppression of ATPA7 and CTR1 protein levels by RNAi, parasite infection increases in macrophages, revealing a host protective role for Cu transporters against Leishmania parasites. Moreover, Cu supplementation to diet reduces Leishmania lesions in infected mice, whereas treatment with Cu chelator promote lesions and parasite loads. Finally, systemic mobilization of Cu from the heart during infection might provide Cu for helping the control of infection. The findings are novel and interesting for the understanding of pathogen-host interplay in a model of a relevant disease. Reproducibility was discussed below.
Specific points:
1) The text seems unbalanced, in special in results, which bear information repeated from introduction, excessively detailed method contents, and extensive discussion. This section reading can be improved by moving information to introduction, methods, and discussion.
2) It is important to add a statistic subsection in methods to better understanding of analyses.
3) The mouse experiment used 3 mouse/group, what is comprehensible because of the number of control uninfected groups, however an n=3 does not allow normality test to define the statistical analysis. One possible solution is to repeat the experiment with a lower number of groups: 3 infected groups and only a non-infected group to improve reproducibility and data analyses.
4) Data availability: a raw data file would make easier to readers to assess the meaning of samples (n) in legends and the reproducibility of data.
Minor points:
1) There is a problem with colors in figures that preclude a better analyses of data distribution. For example, in figure 1b, black points over black lines, red points over red color.
2) There is an inaccurate statement about increase in copper content in Fig. 1c which is non-significant. There is no reason to value it as an increase if it fails analyses. Otherwise, increasing n would allow better assessment of this issue.
3) There is no section of authorship attributing type of participation to individuals.
4) Discussion ends abruptly without a perspective of how these findings impact on the field of infectious diseases.

Reviewer #2 (Remarks to the Author):

The authors put forth a manuscript suggesting copper homeostasis, and specifically pathways using the copper ATPase ATP7a and the copper importer CTR1, is critical for control of Leishmania parasites. The manuscript shows that L. major infection leads to ATP7a degradation and diminished CTR1, and the authors suggest the parasite alters these molecules to lower host-induced copper stress, as copper is generally antimicrobial. The major finding and strength of the manuscript is that BALB/c mice infected with L. major fed a high copper diet exhibit smaller lesions, and mice fed a copper limiting diet had larger lesions, compared to controls. While this reviewer acknowledges great amount of effort put forth by the authors, overall, the findings are more of an association rather than a causation with many gaps remaining.

Major comments:

1. The study is based on Fig 1 where the authors state copper and ATP7a colocalizes with Leishmania parasites (based on parasite DNA). The parasites are unexpectedly small and extremely difficult to see in the images provided (Fig 1A). It is not clear how the authors measured the dye on the Lm fraction (Fig 1B). So, line 148-149 not supported by the observations. Is it possible to visualize or separate out the Lm+ compartments or to confirm with fluorescent parasites that there is indeed colocalization with copper and ATP7a? Additionally, from these images it appears a lot of copper is going to non Leishmania compartments/vesicles.
2. The levels of copper in the skin under different diet conditions was not assessed basally or with infection. Additionally, the ATP7a and CTR1 data from the skin infection site is missing. This information helps to connect the in vitro findings with the in vivo results as presently there is a disconnect in the story.
3. The numbers of viable parasites in the skin in vivo was not assessed. LDAs should be performed to complement PCR results as this is the gold standard in the Leishmania field to assess parasite burdens in tissue.
4. The number of times the experiment has been repeated in Fig 6 should be mentioned
5. Can the authors explain why BALB/c mice did not have lesions at 6 weeks post infection with L. major. This is surprising compared with the rest of the Leishmania field.
6. While the in vitro studies use macrophages, it is not clear if the in vivo results following high or low copper diets involve macrophages. The diets would have systemic affects as seen by heart and liver data.
7. The authors mention ‘fractions’ but it is not clear what fractionation methods were performed and when. It would be helpful to separate out Lm+ compartments to show associated ATP7a and CTR1 association (i.e. co-precipitation, co-localization, etc) to support the parasite involvement in decreasing ATP7a and CTR1 expression. Do they mean fractionation of the images? In line 164, what is residual fractionation? In lines 468-473, it is not clear what figures are being referenced for separating phagolysosomal from golgi ATP7a.
8. The transition and rationale from one experiment to the next needs to be more straightforward as the current state of the manuscript is difficult to follow.
9. Typically in Leishmania studies with macrophages, an MOI of 5:1 is used. Here, 30:1 is used. Can the authors explain the high atypical infection dose? Are the results consistent with a smaller dose like 5:1 or even 10:1 MOI?
10. The conclusions state causation but the data are more associations. Examples: lines 266-276;

lines 340-343 and 366-367 where it is suggested that infection reduces copper uptake but there is no change in total copper in Fig. 1; lines 365-367; for example) host N-glycanase is elevated with infection by transcript but the enzyme activity or blockade looking for differences in western blot bands of ATP7a and CTR1 was not investigated
11. The role of copper homeostasis in the wound healing function outside of parasite control has not been considered in discussion

Minor comments:
-in intro, not always clear if talking about host or pathogen molecules. Example line 104
-line 116 and 120 seems to contradict one another – is host ATP7a trafficking from golgi to Lm+ compartment or is ATP7a decreased? Or both? Later data clarifies this point, but the intro is confusing
-the manuscript is hard to follow when the reason for using each marker (i.e LAMP, NA K-ATP, etc) is not defined.
-line 153 is too strongly worded
-it is not clear what is being compared in results text.
-the conclusions drawn from figure 1 in lines 171-173 is an overstatement as parasite burdens have not been measured at this point in the study. 'copper uptake…a mechanism to facilitate parasite killing'.
-line 175: it is difficult to see the parasite in Fig 1 so concerning to say copper is 'channelized' to Lm+ compartments
-the studies were performed in a mouse macrophage cell line? Are the results specific to cell lines or are similar results found in murine bone marrow-derived macrophages?
-speculation is found throughout the results section. These statements should be moved to the discussion. Example lines 206-211
-line 286 should reference supp figs
-line 306 not clear fold changes references Fig 4B or Fig 4C
-lines 379-385: too much technical detail for results section and can move to materials and methods
-Fig S6 references in results but do not match
- the role of copper homeostasis in the wound healing function or other factors contributing to disease like inflammation or damage instead of parasite control has not been considered; this would change 'combat' statement of lines 396 and 416 and 503
-elevated transcripts like COMMD1 in line 457 do not definitively show function
-no statistics section in M&M
-fig 1 legend says 12 hours but text says 3 hours
-sample size gives number but not clear what this count is for – macrophages? Example line 891, 954, etc
-in figure legends, it would be helpful to state what is being compared because there are not lines over the bar in the figures to show groups being compared
-why do the authors believe there is no difference in parasites with the ATOX1 knock down?

Response to Reviewers' comments:

Reviewer #1:

1) The text seems unbalanced, in special in results, which bear information repeated from introduction, excessively detailed method contents, and extensive discussion. This section reading can be improved by moving information to introduction, methods, and discussion.

Response: We thank the reviewer the valuable suggestion. We have tried to balance out the text in this revised version, particularly, in the Results section which had repetitive information that can fit in Introduction, Methods and Discussion. We have provided more direct and straightforward rationale to move from one experiment to the next in the Results section. We think this will improve the overall readability of the manuscript. The edited text is highlighted in yellow.

2) It is important to add a statistic subsection in methods to better understanding of analyses.

Response: We have added a statistics subsection in methods. The added text is highlighted in yellow. (Line. 695-698)

3) The mouse experiment used 3 mouse/group, what is comprehensible because of the number of control uninfected groups, however an n=3 does not allow normality test to define the statistical analysis. One possible solution is to repeat the experiment with a lower number of groups: 3 infected groups and only a non-infected group to improve reproducibility and data analyses.

Response: We appreciate the concern of the reviewers. We have now repeatedly the mouse experiment where another set of 3 mouse/group has been used for all six conditions (n=6). The revised figure is now Fig.6. The legends to the fig have also been edited (Line.1076-1109).

4) Data availability: a raw data file would make easier to readers to assess the meaning of samples (n) in legends and the reproducibility of data.

Response: We have now uploaded raw data files in excel format (named according to figure numbers).

Minor points:
1) There is a problem with colors in figures that preclude a better analyses of data distribution. For example, in figure 1b, black points over black lines, red points over red color.

Response: We agree with the reviewer and have changed the figures which now have different colors in data points and plots.

2) There is an inaccurate statement about increase in copper content in Fig. 1c which is non-significant. There is no reason to value it as an increase if it fails analyses. Otherwise, increasing n would allow better assessment of this issue.

Response: We agree with the reviewer and have rectified the statement in relation to Fig1C (line 133-135). However, in Fig S2B, we observe a significant increase in copper uptake at later stages of infection (15hr-18hr) and have mentioned it in the discussion section (line 175-177). The new text is highlighted in yellow.

3) There is no section of authorship attributing type of participation to individuals.

Response: We have now added an authorship contribution statement in the manuscript (line.721-725).

4) Discussion ends abruptly without a perspective of how these findings impact on the field of infectious diseases.

Response: We agree with and reviewer and have added an entire section on the implication of this study in the field of infectious diseases. We have rewritten the parts and we have discussed in details about previous studies in the field and how our study adds to the repertoire of knowledge that can be utilised in future. The new text is highlighted in yellow (line no.473-503:)

Reviewer #2 (Remarks to the Author):

Major comments:

1. The study is based on Fig 1 where the authors state copper and ATP7a colocalizes with Leishmania parasites (based on parasite DNA). The parasites are unexpectedly small and extremely difficult to see in the images provided (Fig 1A). It is not clear how the authors measured the dye on the Lm fraction (Fig 1B). So, line 148-149 not supported by the observations. Is it possible to visualize or separate out the Lm+ compartments or to confirm with fluorescent parasites that there is indeed colocalization with copper and ATP7a? Additionally, from these images it appears a lot of copper is going to non-Leishmania compartments/vesicles.

Response: In the revised Fig 1A, we are now showing the parasites in blue and CF4 in magenta with arrows indicating their colocalization. Specifically, insets are provided for clarity of the data (mentioned in legends to fig section: line 966-967).
In Fig 1B, to determine colocalisation of the dye with *Leishmania major* (blue), macrophage nuclei (blue) were omitted from analysis. For omitting macrophage nuclei, 'Analyze particle' plugin was used. 'Otsu' thresholding was applied on DAPI (blue) channel and nucleus was detected keeping the size cut-off of >1000 pixel and circularity 0.04-1.00 and cleared subsequently. Images containing only *Leishmania* nuclei are then used in colocalization with CF4. The ImageJ macro codes for the analysis is available in https://github.com/saps018/Leishmania-host-copper. We have now added this detail in Material and Method's Image analysis section (line. 683-689)
We applied the same 'Otsu' thresholding while measuring ATP7A and CTR1 on *L. major* (Figs 1G and 5C).
For ATP7A, we agree that there is not a massive shift from Golgi to pathogen compartment (now discussed in text). Rather, it is a dispersal from the Golgi (but unlike copper treatment, ATP7A does not reach the plasma membrane) (line. 144-147). Some of it ends up in majority of the *Leishmania* compartments which are present in limited numbers (Fig 1G). The edited text and legends are highlighted in yellow.

2. The levels of copper in the skin under different diet conditions was not assessed basally or with infection. Additionally, the ATP7a and CTR1 data from the skin infection site is missing. This information helps to connect the in vitro findings with the in vivo results as presently there is a disconnect in the story.

Response: We agree that having the data from infection sites will be definitely useful in this study. We have now added copper measurements data from the skin under different diet conditions basally or with infection. Interestingly, copper content is high in all cases of infection that includes (a) basal + infected (b) copper + infected and (c) TTM + infected (**new Fig 6I**). We have also added ATP7A and CTR1 data from the skin infection from 15-week post-infection. In both cases, proteins levels go up upon infection (new Fig 6J). The edited text is highlighted in yellow (line no.370-376).

3. The numbers of viable parasites in the skin in vivo was not assessed. LDAs should be performed to complement PCR results as this is the gold standard in the Leishmania field to assess parasite burdens in tissue.

Response: We have added LDAs from the footpad infection studies to assess the number of parasites (Fig 6E) and in the text (line.363-364). We have added appropriate reference Titus et al, Parasite Immunol. 1985 Sep;7(5):545-55. of LDA in Material and Method section (654-655). Copper treated group had low parasite load in their footpad as evident from limiting dilution assay and kDNA C_t value (Fig. 6E and 6F) The edited text is highlighted in yellow (line no. 363-364).

4. The number of times the experiment has been repeated in Fig 6 should be mentioned

Response: The experiment in Fig6 has been repeated twice, each time with 3 BALB/c mice for all the conditions (n=6). It is now mentioned in the Legend to the fig6.

5. Can the authors explain why BALB/c mice did not have lesions at 6 weeks post infection with L. major. This is surprising compared with the rest of the Leishmania field.

Response: We agree that the onset of lesions was delayed. We used 1×10^6 late log-phase *L. major* for footpad infection. As compared to stationary phase, parasites exhibit low virulence during the logarithmic growth phase. The high ratio of metacyclic parasites in the stationary growth phase contributes to high virulence (da Silva R, Sacks DL. Metacyclogenesis is a major determinant of Leishmania promastigote virulence and attenuation. Infect Immun. 1987 Nov;55(11):2802-6. doi: 10.1128/iai.55.11.2802-2806.1987. PMID: 3666964; PMCID: PMC259980). In a previous study by our group, we recorded a similar phenomenon where the onset of lesion starts ~6 weeks. (Paul et al, J Biol Chem. 2022 Feb;298(2):101539. doi: 10.1016/j.jbc.2021.101539)

6. While the in vitro studies use macrophages, it is not clear if the in vivo results following high or low copper diets involve macrophages. The diets would have systemic affects as seen by heart and liver data.

Response: *Leishmania major* footpad infection occurs following their engulfment by macrophages. Inside macrophages, the intracellular amastigotes grow in number and burst out, infecting other macrophages in the area. As per reviewer's suggestion, we measured copper content in the footpad (Fig 6I). The edited text is highlighted in yellow (line no.370-376). We observe that high and low copper diet actually effect their copper content. In cases of infection, where footpad lesions are comprised of infected macrophages, we observed heightened copper level (Fig 6I). Not only that, high copper diet resulted in heightened copper level whereas TTM treatment resulted in reduced copper levels.

7. The authors mention 'fractions' but it is not clear what fractionation methods were performed and when. It would be helpful to separate out Lm+ compartments to show associated ATP7a and CTR1 association (i.e. co-precipitation, co-localization, etc) to support the parasite involvement in decreasing ATP7a and CTR1 expression. Do they mean fractionation of the images? In line 164, what is residual fractionation? In lines 468-473, it is not clear what figures are being referenced for separating phagolysosomal from golgi ATP7a.

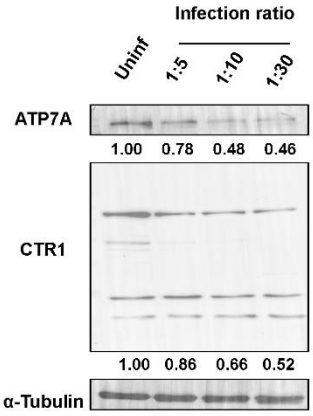
Response: We apologize for the confusion arising due to usage of the term 'fraction'. No, we have not performed fractionation methods. We agree the use of the word "fractions" can be misleading and have changed it in the text.
We have provided colocalisation data between Lm+ compartments (in blue) with ATP7A and CTR1(in green) (Figs. 1G and 5C). (146-148 and 302-304.)
Fraction or residual fraction implies to the differentially localised ATP7A (one in Golgi and the other which has trafficked out in response to infection). We have changed these terms in the text.
We have now provided the figures (Fig 1F, 1G and 1H) which are being referenced in the text for separating phagolysosomal ATP7A from Golgi ATP7A. (line no. 140-152)

8. The transition and rationale from one experiment to the next needs to be more straightforward as the current state of the manuscript is difficult to follow.

Response: We have tried to balance out the text in this revised version, particularly, in the Results section which had repetitive information that can fit in Introduction, Methods and Discussion. Now, as per Reviewer's suggestion, we have provided more direct and straightforward rationale to move from one experiment to the next in the Results section. We think this will improve the overall readability of the manuscript. All edited texts are highlighted in yellow.

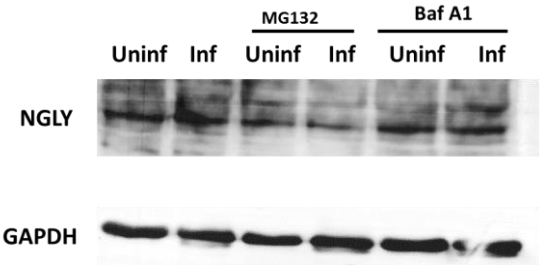
9. Typically in Leishmania studies with macrophages, an MOI of 5:1 is used. Here, 30:1 is used. Can the authors explain the high atypical infection dose? Are the results consistent with a smaller dose like 5:1 or even 10:1 MOI?

Response: We have now performed infection studies at lower MOIs and compared with the existing MOI 30:1. We observed similar changes in ATP7A and CTR1 at the lower MOIs but we observed a distinct and clear phenotype for MOI 30:1 (Particularly for CTR1). Figure is provided below. It seems that the higher initial dose of *L. major* effects the host copper transporters to a greater extent.



10. The conclusions state causation but the data are more associations. Examples: lines 266-276; lines 340-343 and 366-367 where it is suggested that infection reduces copper uptake but there is no change in total copper in Fig. 1; lines 365-367; for example) host N-glycanase is elevated with infection by transcript but the enzyme activity or blockade looking for differences in western blot bands of ATP7a and CTR1 was not investigated.

Response:
Lines: 266-276:
We thank the reviewer the suggesting the experiments with N-glycanase, that would confirm its role. We checked its protein levels upon infection and blocked the two degradation pathways (3 hours). Figure is attached below. Since, we did not find any significant changes, we agree with the reviewer that its transcript level increase may be association rather than causation. We, therefore, decided to take out the transcript data from the manuscript.



Lines 340-343 and 366-367:
Although CTR1 endocytosis occurs in both Copper treated and Infection conditions, the post infection treatments suggest that a copper independent trafficking exists during infection (CTR1 that endocytosed upon infection, do not respond to copper or copper chelator treatments). The immunoblot additionally supports the claim as the band patterns are different between copper treated and Infection conditions. We have now added a reference for copper independent CTR1 trafficking which actually provides the rationale to move into the next experiment of checking CTR1 Cys redox status.

Macrophage CTR1 expression increases upon activation by LPS and IFN-γ resulting in increased copper uptake (*White C, Lee J, Kambe T, Fritsche K, Petris MJ. A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. J Biol Chem. 2009 Dec 4;284(49):33949-56. doi: 10.1074/jbc.M109.070201*). We agree that in Fig1C, there is no change in total copper uptake. Total internal copper is tightly regulated by balance of CTR1 uptake and ATP7A export. The internal copper levels did not decrease below the basal level upon infection-triggered CTR1 endocytosis because it was balanced out by reduced copper export. Rather, we argue that if CTR1 was not downregulated by *L. major* then copper uptake would have increased as observed in the above-mentioned study. In Fig S2B, we observe a significant increase in copper uptake at later stages of infection (15hr-18hr) that corroborates with the recovery of CTR1 levels. Since ATP7A receives copper from CTR1 via ATOX1, we believe that CTR1 downregulation effectively lowers copper loading on ATP7A at early infection stage (even though there is no decrease in total internal copper level).

We agree to the reviewer's comments and made necessary changes in the text (in line no.133-135; 175-177 as suggested by the reviewer).

11. The role of copper homeostasis in the wound healing function outside of parasite control has not been considered in discussion

Response: We thank the reviewer for this valuable suggestion and now we have discussed the role of copper homeostasis in details in the wound healing function and inflammatory cell recruitment at sites of infection (line.494-499). We feel that this significantly adds the overarching, beneficial role of copper particularly against Cutaneous Leishmaniasis.

Minor comments:
-in intro, not always clear if talking about host or pathogen molecules. Example line 104

Response: In introduction, we have now made necessary changes that makes it clear whether we are talking about host or pathogen. Particular changes have been made in line 87-90 in the revised text and highlighted in yellow.

-line 116 and 120 seems to contradict one another – is host ATP7a trafficking from golgi to Lm+ compartment or is ATP7a decreased? Or both? Later data clarifies this point, but the intro is confusing.

Response: We agree that these statements can indeed be confusing. In those parts, while mentioning ATP7A decrease upon infection (introducing the statement for the first time in the manuscript), we have specifically used "early time-points". This is followed by a line where we are mentioning about ATP7A trafficking from Golgi to Lm+ compartment, we have added "late stages". The changes are now in line no. 104-108.

-the manuscript is hard to follow when the reason for using each marker (i.e LAMP, NA K-ATP, etc) is not defined.

Response: We agree that each marker used in the study should be defined. We have made sure to particularly define each one of them (i.e LAMP, Na,K-ATPase, etc) in context to the experiments. Line no.140-143 and 292-295.

-line 153 is too strongly worded

Response: We agree with the reviewer and have rectified the statement in relation to Fig1C (line 133-135). However, in Fig S2B, we observe a significant increase in copper uptake at later stages

of infection i.e., 15hr-18hr (line. 175-177) and have mentioned it in the discussion section. All changes are highlighted in yellow.

-it is not clear what is being compared in results text.

Response: We have now specifically stated what is being compared in the text and in the figure legends of each graph and plots present in the manuscript.

-the conclusions drawn from figure 1 in lines 171-173 is an overstatement as parasite burdens have not been measured at this point in the study. 'copper uptake...a mechanism to facilitate parasite killing'.

Response: We agree that at this point in the manuscript, it is an overstatement. In the revised manuscript, we have referred to a study that shows that macrophage utilises exogenous copper for more efficient intracellular pathogen killing (White C, Lee J, Kambe T, Fritsche K, Petris MJ. A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. J Biol Chem. 2009 Dec 4;284(49):33949-56. doi: 10.1074/jbc.M109.070201). In the text, we now speculate that such response may be at play upon exogenous copper treatment on *L. major* infected macrophages. (Line no.158-161)

-line 175: it is difficult to see the parasite in Fig 1 so concerning to say copper is 'channelized' to Lm+ compartments.

Response: We have now added magnified insets showing *Leishmania* nuclei and CF4 dye colocalising with quantification. We have replaced the word 'channelized' with accumulation.

-the studies were performed in a mouse macrophage cell line? Are the results specific to cell lines or are similar results found in murine bone marrow-derived macrophages?

Response: Yes, the studies were performed in a mouse macrophage cell line. We have performed the same study in peritoneal macrophages from BALB/c mice, where we obtained similar results (S6A).

-speculation is found throughout the results section. These statements should be moved to the discussion. Example lines 206-211

Response: We agree with the reviewer and rectified it in this revised version of the manuscript. We have moved speculative statements from the Results section to discussion. We thank the reviewer for the suggestion as this will improve the overall readability of the manuscript.

-line 286 should reference supp figs

Response: Yes, we have referred to the Figure S4 (Line. 248-249).

-line 306 not clear fold changes references Fig 4B or Fig 4C

Response: We thank the reviewer for pointing this out. The statement refers to Fig4B which we now have rectified in our manuscript. (line no. 267-269)

-lines 379-385: too much technical detail for results section and can move to materials and methods

Response: We have now moved the technical detail to materials and methods section in our revised manuscript.

-Fig S6 references in results but do not match

Response: We thank the reviewer for pointing this out. We have altered the figure numbers for Haemoglobin and copper measurement to maintain their chronology in the text. We have also referred to Figure S6D (now S6E) in the revised text. (Line no. 391-393)

- the role of copper homeostasis in the wound healing function or other factors contributing to disease like inflammation or damage instead of parasite control has not been considered; this would change 'combat' statement of lines 396 and 416 and 503

Response: We agree with the reviewer that copper homeostasis and regulation have profound roles in wound healing and recruitment of inflammatory cells to the wound site (Das, A., Sudhahar, V., Chen, GF. et al. Endothelial Antioxidant-1: a Key Mediator of Copper-dependent Wound Healing in vivo. Sci Rep 6, 33783 (2016). https://doi.org/10.1038/srep33783). We have also seen studies where copper has accumulated in intracellular pathogenic compartments inside macrophages. Pathogenic copper exporters become pivotal for their survival suggesting the cytotoxic effect of elevated copper (Paul R, Banerjee S, Sen S, Dubey P, Maji S, Bachhawat AK, Datta R, Gupta A. A novel leishmanial copper P-type ATPase plays a vital role in parasite infection and intracellular survival. J Biol Chem. 2022 Feb;298(2):101539. doi: 10.1016/j.jbc.2021.101539) (White C, Lee J, Kambe T, Fritsche K, Petris MJ. A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. J Biol Chem. 2009 Dec 4;284(49):33949-56. doi: 10.1074/jbc.M109.070201). These points have been mentioned and discussed in details in the discussion section (line no.490-499).

We believe that copper is beneficial in more than one way. We agree with the reviewer and have discussed the role of copper homeostasis particularly with respect to inflammation and damage. We have changed "combat" in the above-mentioned lines, rather we have mentioned about the multimodal effects of elevated systemic copper. (line no. 359, 385 and 463-464)

-elevated transcripts like COMMD1 in line 457 do not definitively show function

Response: Yes, we do not observe change at the protein levels of COMMD1. We are unsure why its transcript is elevated. We have now discussed it in the manuscript in the discussion section and modified the above-mentioned line. (Line no. 424-427)

-no statistics section in M&M

Response: We have added a statistics section in M&M (line no.695-698).

-fig 1 legend says 12 hours but text says 3 hours

Response: We thank the reviewer for pointing this out. It is 12 hours and we have altered it in the text.

-sample size gives number but not clear what this count is for – macrophages? Example line 891, 954, etc

Response: Yes, the count is for the macrophage. In the revised version, we have mentioned macrophage counts in the legend section. All edits are highlighted in yellow (Fig1 and Fig5 legends to figure).

-in figure legends, it would be helpful to state what is being compared because there are not lines over the bar in the figures to show groups being compared.

Response: We thank the reviewer for pointing this out. We have now specifically stated what is being compared in the figure legends of each graph and plots present in the manuscript. All edits are highlighted in yellow

-why do the authors believe there is no difference in parasites with the ATOX1 knock down?

Response: It is very important point raised by the reviewer. We believe that the initial stages of *Leishmania* infection followed by its colonisation in macrophage under in vitro conditions is majorly determined by the amount of copper accumulation in phagosomal compartments.

We cannot exclude the possibility that other proteins apart from ATP7A can contribute to this copper accumulation. CTR1 being the primary copper importer hence becomes crucial in determining parasite load as it relays copper to multiple pathways.

For ATOX1 knocked down, interestingly we did not observe changes in parasite load (Fig. 4B and 4C). Particularly given its role in mediating wound healing, the results were surprising. We believe that the infection time-point (12hr) and in vitro conditions might have led to such results. Under *in vivo* conditions and under a relatively long incubation time (4-6weeks), ATOX1 may exhibit a copper-dependent effect on *L. major* as both nuclear transcription factor and cytosolic copper chaperone (Das, A., Sudhahar, V., Chen, GF. et al. Endothelial Antioxidant-1: a Key Mediator of Copper-dependent Wound Healing in vivo. Sci Rep 6, 33783 (2016). https://doi.org/10.1038/srep33783.)

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors solved the previous raised scientific concerns in this revised manuscript version. A specialized scientific editing throughout the text is still needed to improve ms readability.

Reviewer #2 (Remarks to the Author):

The added data to figures 1, 6 and S6 strengthen the revised manuscript. This reviewer recognizes the immense amount of work that went into the revision, and applauds the efforts of the authors. There are some minor modifications needed for the newly added text.

Fig 1 legend: there is no green in Fig. 1A as the legend says. What does sample size of 12 mean for a cell line? (the experiment repeated 12 times? If so, there is not 12 dots in quantification bar chart).

Lines 140-152: this part of the revised text is difficult to follow. This text needs to be simplified and streamlined. Also, some sentences in this new text are not clear (ex. "We observed further dispersal of ATP7a in vesicles as compared to only infection.") – further than what? This text section is also out of order – for instance line 145 describing experiment design should go before results in line 140. In line 144 the authors state "ATP7A trafficking was Leishmania infection-specific"… but uninfected cells given copper also increased the number of ATP7A+ vesicles, so ATP7A is not infection-specific. It would be more appropriate to say infection increases rather than "infection-specific".

Lines 158-161 and lines 374-376 are not results and should be included in the discussion.

Lines 500-503 do not fulfill the grammatical definition of a paragraph.

Reviewers' comments:

Reviewer #1:

Comment: The authors solved the previous raised scientific concerns in this revised manuscript version. A specialized scientific editing throughout the text is still needed to improve ms readability.

Response: We thank the reviewer for his suggestion. We have made multiple edits to improve the readability of the manuscript. All the changes are highlighted in yellow.

Reviewer #2:

Comment: The added data to figures 1, 6 and S6 strengthen the revised manuscript. This reviewer recognizes the immense amount of work that went into the revision, and applauds the efforts of the authors. There are some minor modifications needed for the newly added text. Fig 1 legend: there is no green in Fig. 1A as the legend says. What does sample size of 12 mean for a cell line? (The experiment repeated 12 times? If so, there is not 12 dots in quantification bar chart).

Response: We apologise for the inadvertent typographical error in the legend. There is no green channel in Fig1A and changes have been made and highlighted in the legend section.
Sample size of 12 means 12 individual infected macrophage cells where the fraction of dye (CF4 and control dye) on intracellular *Leishmania* nuclei has been measured. In Fig. 1B, upon verification, we do observe 12 dots in the graph.

Comment: Lines 140-152: this part of the revised text is difficult to follow. This text needs to be simplified and streamlined. Also, some sentences in this new text are not clear (ex. “We observed further dispersal of ATP7a in vesicles as compared to only infection.”) – further than what? This text section is also out of order – for instance line 145 describing experiment design should go before results in line 140. In line 144 the authors state “ATP7A trafficking was Leishmania infection-specific”… but uninfected cells given copper also increased the number of ATP7A+ vesicles, so ATP7A is not infection-specific. It would be more appropriate to say infection increases rather than “infection-specific”.

Response: We appreciate the reviewer’s concern. We have now simplified this section which is highlighted in the text. We have described the experiment first. Then, we go through the observations one by one, finally culminating in the overall result. We have replaced the line “We observed further dispersal of ATP7A in vesicles as compared to only infection.” We mentioned that ATP7A traffics from Golgi in infection even in absence of external copper treatment. We have also mentioned about increased ATP7A trafficking upon copper treatment irrespective of infection. We are not using the term “infection-specific” in our revised version.

Comment: Lines 158-161 and lines 374-376 are not results and should be included in the discussion.

Response: We agree with the reviewer and removed these lines from the result section and merged them in the discussion.

Comment: Lines 500-503 do not fulfil the grammatical definition of a paragraph.

Response: We have now merged this section with the previous paragraph and highlighted it.