Human microsporidian pathogen *Encephalitozoon intestinalis* impinges on enterocyte membrane trafficking and signaling
Juan Flores, Peter M. Takvorian, Louis M. Weiss, Ann Cali and Nan Gao
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*MS TITLE: Human microsporidian pathogen Encephalitozoon intestinalis infection alters enterocyte landscape and signaling*

AUTHORS: Juan Flores, Peter M. Takvorian, Louis M. Weiss, Ann Cali, and Nan Gao
ARTICLE TYPE: Research Article

We apologize for the slower than average review process in the current pandemic climate, but have now reached a decision on the above manuscript.

To see the reviewers’ reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the ‘Manuscripts with Decisions’ queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers are positive but raise a number of criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using ‘Tracked changes’ in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the ‘Response to Reviewers’ box. Please attend to all of the reviewers’ comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.
Reviewer 1

Advance summary and potential significance to field

Microsporidia are a large phylum of obligate intracellular parasites. Many species of microsporidia infect humans where they are responsible for disease and occasionally death, especially in immunocompromised individuals. To better understand the impact of microsporidia on human cells, the authors infected Caco2 cells with Encephalitozoon intestinalis, and showed that these culture cells can be used to recapitulate the parasite’s life cycle. Using transmission electron microscopy the authors describe this life cycle and quantify both nuclear and mitochondrial morphological changes that occur upon infection. The authors also do RNA-seq on infected cells and show enrichment of several categories of genes including mitochondrial pathways. This is an interesting paper that couples high-resolution imaging of cell morphology and transcriptional profiling to understand the impact of microsporidia infection on human cells.

Comments for the author

Minor points:
1. The pairs of the two uninfected RNA-seq samples look different from each other and it’s not clear from the methods if they were treated any different. The methods appear to only describe 4 total samples (2 infected and 2 uninfected), so it’s not clear where the seven samples for RNA-seq came from. This should be clarified in the methods.
2. The enrichment score vs genes regulated graphs should be described in the figure legends. I have never encountered such a representation and I am not sure how to interpret them. For several of these graphs the font size is also different between panels of the same figure.
3. Changing the scales on the heat maps to units of fold change would be useful, rather than just having them defined as row min and row max.
4. Figure 4I: Should add a statistical comparison
5. Figure 3E: CT is not defined. Either write “control” or define in figure legend.
6. The figure legends contain several sentences that appear to contain mistakes and need to be corrected. Figure 3 “(F) GSEA shows enhanced expression of the same genes described in”. Figure 4 “(G&H) Heat of expression of genes described in D and E respectively.”
7. Figure 2C: The z-score scale is not explained and why the genes selected are only between -1.5 and 1.5 is confusing.

Reviewer 2

Advance summary and potential significance to field

This paper reports bulk RNA sequencing data for the host cell response of Caco2 cells infected with the microsporidian E. intestinalis. This is the first report of RNA seq data for cells infected with E. intestinalis, and Caco2 cells are a good model for this species. As such, the data are valuable to the field. Some TEM analyses are also presented, in conjunction with the RNA seq data. While the RNA seq data itself are valuable, and may lay the groundwork for interesting future work, we feel that much of the data are over-interpreted, and the conclusions are not justified by the data presented. In order to make these conclusions, followup mechanistic work will be required. Alternatively, much of the interpretation could be moved to the discussion section, also presenting alternative hypotheses.

Major comments
1) As stated above, our main concern with this manuscript is over-interpretation of the data, based on what is presented in the manuscript. One example is the authors discuss SNARE-mediated fusion between the parasitophorous vacuole and host membranes (nuclear and mitochondrial). This may be a possibility, but follow-up experiments would be required to assess this. These include modulating host-cell SNARE activity using inhibitors or genetic tools. The examples of TEM images are not sufficient to conclude molecular mechanisms. We understand that manipulation on the parasite side may not be feasible due to a lack of tools in the field. However, to test hypotheses
originating from the RNA seq data, manipulations on the host side can be made using standard techniques, to follow up on the proposed mechanisms. We recommend that either these follow-up experiments are performed and the results presented, or, alternatively, these (very plausible) ideas could be discussed in the discussion section, so as not to mislead the reader.

2) E. intestinalis has already been successfully propagated in Caco2 cells previously, showing that this species can establish an active infection in these cells (Leitch et al, Infection and Immunity, 2005, p. 7697-7704). Since this is the main point of Fig. 1, we feel this paper should be referenced and discussed. The new data presented here compared with the previous publication are the TEM images of germinating spores in the PV.

3) The observation that infection results in upregulation of genes involved in proliferation is intriguing and counter-intuitive, as previously it has been hypothesized that microsporidia infection results in diminished growth, and reduction in host energy consumption activities, including proliferation, was observed in a different species (Watson et al. BMC Genomics (2015) 16:983). Can this be explored further? For example can this phenotype be confirmed by performing growth/proliferation assays/ FACS analysis of growth profiles with infected cells?

4) For figures such as Fig. 3I, we found showing the heatmap to be confusing, and it could be misleading, due to the differences in replicates, which is quite large. We suggest showing volcano plots instead in these cases, which would better take into account the reproducibility in the replicates, and help the reader to focus on consistent differences.

Minor comments
1) We suggest that the title be more specific to the findings of the paper, as any infection is likely to change the transcriptome of the host cell in some way.

2) We did not follow the following sentence in the abstract: “Initially, Encephalitozoon intestinalis was identified as the causative agent responsible for diarrhea, renal failure, and dissemination to other organs in immune compromised individuals.”. The second part seems unconnected to the first, and the authors may have meant to write “a” causative agent instead of “the” causative agent.

3) Please discuss why 14 days post infection is used for RNA-seq. At 21 days post infection (IF data), how many cells are infected? Quantification of this would be helpful in understanding how the methods were developed and specific timepoints chosen

4) Fig. 1 A, D does not have a scale bar and is hard to interpret

5) The authors discuss differentiation of Caco2 cells once they become confluent, and use an example of a TEM image to probe this, which shows the formation of microvilli. Other aspects of differentiation discussed in the text cannot be seen. Perhaps markers to show polarity or additional views from TEM would be helpful to assess this?

6) “Spores both inside and outside the PV” were noted. It is not clear whether the authors mean that spores were phagocytosed, or that mature spores were released from the PV, since E. intestinalis is thought to grow within PVs. Some clarification on this point and TEM to show this would be helpful.

7) Fig. 1G is not referenced in the text, and Figs. 1E and 1F are incorrectly referenced in the text.

8) We did not understand the conclusion that germinated spores are indicative of “auto-infection”. Other hypotheses also seem plausible, such as escape, spread.

9) Fig 3: In figure 3B what is the arrowhead pointing to/ indicating? Are Figures 3B, C and D mislabelled in the text? We had a hard time following Fig. 3E. What are the percentages next to the boxed mitochondria examples indicating? If we understood this correctly, >50% in the control are “abnormal”, which we could not follow.
10) With regard to the denovo biogenesis of mitochondria, while it would be challenging to alter anything related to mitochondrial pathways without affecting the viability of the host cells, TEM images are already available. Can the authors quantify the number of mitochondria in uninfected and infected cells to gain insights into whether the number of mitochondria/cell were increased in the infected cells?

11) Figures might be mislabeled in the text e.g. Fig. 4C in the text should be 4D and Fig. 4D in text should be 4E?

12) With respect to the PV that is being engulfed by the host cell nucleus, do the authors mean ‘we found some PVs with parasites with thicker membrane lining almost completely engulfed by the host nucleus’? In the TEM image, the PV membrane doesn’t look very different, however, it seems that the parasites within the PV however do have thicker membranes.

13) We did not follow how measurements shown in Fig. 4I were made. Could the authors please include this in the methods section?

14) Fig. 5: Please show the same magnification image for Fig. 5A and C, so the reader can compare these easily (lower mag of Fig. 5C would be helpful). Fig. 5B seems to be incorrectly referenced in the text.

15) Please include imaging conditions for fluorescent imaging in the methods

Comments for the author
please see above

First revision

Author response to reviewers' comments

Point-by-point Response (MS ID#: JOCES/2020/253757)

Reviewer 1 Advance Summary and Potential Significance to Field: Microsporidia are a large phylum of obligate intracellular parasites. Many species of microsporidia infect humans where they are responsible for disease and occasionally death, especially in immunocompromised individuals. To better understand the impact of microsporidia on human cells, the authors infected Caco2 cells with Encephalitozoon intestinalis, and showed that these culture cells can be used to recapitulate the parasite’s life cycle. Using transmission electron microscopy the authors describe this life cycle and quantify both nuclear and mitochondrial morphological changes that occur upon infection. The authors also do RNA-seq on infected cells and show enrichment of several categories of genes including mitochondrial pathways. This is an interesting paper that couples high-resolution imaging of cell morphology and transcriptional profiling to understand the impact of microsporidia infection on human cells.

Author’s Response: We appreciate Reviewer 1’s overall positive assessment of our work. We have carefully addressed the remaining points below, and would like to thank the Reviewer for the critiques.

Reviewer 1 Comments for the Author: Minor points:
1) The pairs of the two uninfected RNA-seq samples look different from each other and it’s not clear from the methods if they were treated any different. The methods appear to only describe 4 total samples (2 infected and 2 uninfected), so it’s not clear where the seven samples for RNA-seq came from. This should be clarified in the methods.
Author’s Response: Thanks for raising this question. We have now described in Results and Legend that two independent experiments were performed to obtain infected and uninfected cells. From each experiment, 2 infected and 2 uninfected samples were collected, and a total of 8 samples (4 uninfected and 4 infected) were used for bulk RNA sequencing analysis. The RNA quality of one infected sample did not pass quality control threshold for RNA sequencing. As a consequence, we sequenced 3 infected and 4 uninfected samples, leading to a total of seven samples. The two pairs of uninfected samples looked different, and we believe that this was due to the independent passaging, culturing, and harvesting. However, even with such intra-group variations, all control samples were strikingly different from infected samples as revealed in our analysis.

2) The enrichment score vs genes regulated graphs should be described in the figure legends. I have never encountered such a representation and I am not sure how to interpret them. For several of these graphs the font size is also different between panels of the same figure.

Author’s Response: We have now included the citation and a brief description of Gene Set Enrichment Analysis (GSEA) in the method section. GSEA is designed to analyze a transcriptional signature or a set of genes that characterize a specific pathway or functional category. The leading-edge plot (green curve) shows whether this specific gene set was increased or decreased in the experimental group compared to control group. We have now made all the fonts consistent in size.

3) Changing the scales on the heat maps to units of fold change would be useful, rather than just having them defined as row min and row max.

Author’s Response: We added a supplemental table using edgeR analysis for all differentially changed genes including fold change, p value and adjusted p value.

4) Figure 4I: Should add a statistical comparison

Author’s Response: A statistical analysis has been added to the figure.

5) Figure 3E: CT is not defined. Either write “control” or define in figure legend.

Author’s Response: This is now defined in figure legend.

6) The figure legends contain several sentences that appear to contain mistakes and need to be corrected. Figure 3 “(F) GSEA shows enhanced expression of the same genes described in”. Figure 4 “(G&H) Heat of expression of genes described in D and E respectively.”

Author’s Response: These typo and errors have been corrected.

7) Figure 2C: The z-score scale is not explained and why the genes selected are only between -1.5 and 1.5 is confusing.

Author’s Response: The z-score is a measure of the distance in standard deviations from the plate mean. A sample with a z-score of 0 has the same raw value as the plate mean.

Reviewer 2 Advance Summary and Potential Significance to Field: This paper reports bulk RNA sequencing data for the host cell response of Caco2 cells infected with the microsporidian E. intestinalis. This is the first report of RNA seq data for cells infected with E. intestinalis, and Caco2 cells are a good model for this species. As such, the data are valuable to the field. Some TEM analyses are also presented, in conjunction with the RNA seq data. While the RNA seq data itself are valuable, and may lay the groundwork for interesting future work, we feel that much of the data are over-interpreted, and the conclusions are not justified by the data presented. In order to make these conclusions, follow up mechanistic work will be required. Alternatively, much of the interpretation could be moved to the discussion section, also presenting alternative hypotheses.

Author’s Response: We thank Reviewer 2 for recognizing the valuable contribution of our study, and for raising criticism towards some over-interpretations of our RNA seq data. We have performed additional experiments to follow up some clues generated by our RNA seq data. We have also
removed some interpretations to Discussion. Please see detailed changes below. We hope the reviewer find that these revisions improved the overall quality of the manuscript.

Major comments

1) As stated above, our main concern with this manuscript is over-interpretation of the data, based on what is presented in the manuscript. One example is the authors discuss SNARE-mediated fusion between the parasitophorous vacuole and host membranes (nuclear and mitochondrial). This may be a possibility, but follow-up experiments would be required to assess this. These include modulating host cell SNARE activity using inhibitors or genetic tools. The examples of TEM images are not sufficient to conclude molecular mechanisms. We understand that manipulation on the parasite side may not be feasible due to a lack of tools in the field. However, to test hypotheses originating from the RNA seq data, manipulations on the host side can be made using standard techniques, to follow up on the proposed mechanisms. We recommend that either these follow-up experiments are performed and the results presented, or, alternatively, these (very plausible) ideas could be discussed in the discussion section, so as not to mislead the reader.

Author’s Response: After carefully evaluating Reviewer’s comments, we performed additional experiments to specifically follow up on the hypothesis about mitochondrial changes and about lysosomal function. These new results are now included in Fig. 3K and 5I. In addition, we took Reviewer’s advice and moved the experimental interpretation about SNARE (see below please) to the Discussion section. We hope these responses are acceptable and not perceived as misleading any more.

2) E. intestinalis has already been successfully propagated in Caco2 cells previously, showing that this species can establish an active infection in these cells (+- et al, Infection and Immunity, 2005, p. 7697-7704). Since this is the main point of Fig. 1, we feel this paper should be referenced and discussed. The new data presented here compared with the previous publication are the TEM images of germinating spores in the PV.

Author’s Response: Thanks for this important comment. We are aware of Dr. Gordon Leitch’s outstanding contributions to the study of Microsporidia and especially his group’s extensive work on factors that trigger spore/polar tube activation. In several of our published studies we have used his germination buffers and extensively cited his work. We were aware of both Foucault and Drancourt (2000) and Leitch et al., (2005) utilization of E. intestinalis and Caco-2 cells for spore activation/cell infection studies. The two reports were somewhat contradictory, and focused on the means of initial infection. In light of the reviewer’s suggestion, we revisited the Leitch paper and the Foucault report, and have added both to the introduction and discussion.

3) The observation that infection results in upregulation of genes involved in proliferation is intriguing and counter-intuitive, as previously it has been hypothesized that microsporidia infection results in diminished growth, and reduction in host energy consumption activities, including proliferation, was observed in a different species (Watson et al. BMC Genomics (2015) 16:983). Can this be explored further? For example, can this phenotype be confirmed by performing growth/proliferation assays/ FACS analysis of growth profiles with infected cells?

Author’s Response: Thanks for the excellent suggestion and we cited this paper and indicated that this will be an important direction for future investigation.

4) For figures such as Fig. 3I, we found showing the heatmap to be confusing, and it could be misleading, due to the differences in replicates, which is quite large. We suggest showing volcano plots instead in these cases, which would better take into account the reproducibility in the replicates and help the reader to focus on consistent differences.

Author’s Response: We have followed the Reviewer’s advice, and included volcano plot in Fig. 3H to demonstrate a preferential increase of mitochondria genes.

Minor comments

1) We suggest that the title be more specific to the findings of the paper, as any infection is likely to change the transcriptome of the host cell in some way.
Author’s Response: We have revised the title to emphasize more confined cellular changes by Encephalitozoon intestinalis.

2) We did not follow the following sentence in the abstract: “Initially, Encephalitozoon intestinalis was identified as the causative agent responsible for diarrhea, renal failure, and dissemination to other organs in immune compromised individuals.”. The second part seems unconnected to the first, and the authors may have meant to write “a” causative agent instead of “the” causative agent.

Author’s Response: We agree with the Reviewer and have clarified this sentence.

3) Please discuss why 14 days post infection is used for RNA-seq. At 21 days post infection (IF data), how many cells are infected? Quantification of this would be helpful in understanding how the methods were developed and specific timepoints chosen clarification.

Author’s Response: This was a misunderstanding of the text written, and we apologize for the confusion. The IF data was on 14 days after infection, which was 21 days after seeding the cells. We have made these time points consistent in the revised paper.

4) Fig. 1 A, D does not have a scale bar and is hard to interpret SCALE BAR ADD

Author’s Response: We have added scale bars to the corresponding images.

5) The authors discuss differentiation of Caco2 cells once they become confluent, and use an example of a TEM image to probe this, which shows the formation of microvilli.

Other aspects of differentiation discussed in the text cannot be seen. Perhaps markers to show polarity or additional views from TEM would be helpful to assess this?

Author’s Response: This point is well taken, and we have added a new TEM image showing an elongated cell undergoing differentiation (Figure 1C).

6) “Spores both inside and outside the PV” were noted. It is not clear whether the authors mean that spores were phagocytosed, or that mature spores were released from the PV, since E. intestinalis is thought to grow within PVs. Some clarification on this point and TEM to show this would be helpful.

Author’s Response: This has been clarified in both figure legends and revised text. We also included additional fluorescent images to show individually stained spores inside cells, as well as free spores in the extracellular space (Fig. 1F-G). When cells died after lysis, the spores were often released from the intracellular PV to extracellular space.

7) Fig. 1G is not referenced in the text, and Figs. 1E and 1F are incorrectly referenced in the text.

Author’s Response: We have now corrected these issues. We thank the Reviewer for identifying these problems.

8) We did not understand the conclusion that germinated spores are indicative of “auto- infection”. Other hypotheses also seem plausible, such as escape, spread.

Author’s Response: We have addressed this critique in our revised text. The majority of Microsporia produce “environmental” spores, these are spores that leave the host in feces, urine, secretions, or upon death. These spores can remain in the environment for weeks to years until they infect a new host. The so-called “auto-infective” spores were first described by Nigelli in 1946, as spores that became activated to extrude their polar tube, and to deposit an infective sporoplasm within the host cell. These spores may reinforce infection or spread infection into neighboring cells. It was hypothesized by us that auto-infective spores are responsible for dissemination of infection to other organs or sites within the host (Cali and Takvorian, 2014). In some microsporidian species, two different morphological types of spores are produced.

9) Fig 3: In figure 3B what is the arrowhead pointing to/ indicating? Are Figures 3B, C and D mislabelled in the text? We had a hard time following Fig. 3E. What are the percentages next to the boxed mitochondria examples indicating? If we understood this correctly, >50% in the control are
“abnormal”, which we could not follow.

Author’s Response: We have clarified these panels in revised manuscript. The parasite has a nuclear plaque (NP), which is the name of the microsporidial equivalent of a centriole and their mitochondrial remnant termed the “mitosome” that retains the biosynthetic machinery for iron-sulfur clusters and some transport functions, but is no longer involved in ATP production (Williams et al., 2014). The percentages in Fig. 3E indicated percentage of cristae integrity. We clarified in revised Figure Legend that 100% refers completely normal cristae structure while 0% corresponds to the absence of cristae in mitochondria. The panel actually showed the vast majority of control mitochondria were normal.

10) With regard to the denovo biogenesis of mitochondria, while it would be challenging to alter anything related to mitochondrial pathways without affecting the viability of the host cells, TEM images are already available. Can the authors quantify the number of mitochondria in uninfected and infected cells to gain insights into whether the number of mitochondria/cell were increased in the infected cells?

Author’s Response: Thanks for this excellent comment. We have performed new experiments to stain mitochondria. We found there were increased mitochondrial number and mitochondrial ROS production (Fig. 3K).

11) Figures might be mislabeled in the text e.g. Fig. 4C in the text should be 4D and Fig. 4D in text should be 4E?

Author’s Response: These have been corrected. Thanks for identifying the mislabeling.

12) With respect to the PV that is being engulfed by the host cell nucleus, do the authors mean ‘we found some PVs with parasites with thicker membrane lining almost completely engulfed by the host nucleus’? In the TEM image, the PV membrane doesn’t look very different, however, it seems that the parasites within the PV however do have thicker membranes.

Author’s Response: Sorry for any confusion, we have now clarified this in the text and figure legends. The PV membrane remains fairly consistent throughout the parasite development and host nucleus envelopment. The “early” proliferative cells (meronts) have a typical “thin” membrane and develop along the inner edge of the PV membrane. They remain attached until sporogony starts and the cell disengages from the PV membrane and is “free” inside the PV. At the start of sporogony, electron dense material is deposited on the outer membrane of the cell (sporont) which then has a “thick” membrane. The deposition of material will continue as the cell becomes a sporoblast and eventually a spore.

13) We did not follow how measurements shown in Fig. 4I were made. Could the authors please include this in the methods section?

Author’s Response: We have provided a schematic diagram in Fig. 4E to demonstrate how we measure the nuclear indentation.

14) Fig. 5: Please show the same magnification image for Fig. 5A and C, so the reader can compare these easily (lower mag of Fig. 5C would be helpful). Fig. 5B seems to be incorrectly referenced in the text.

Author’s Response: We have now provided images with matching magnifications.

15) Please include imaging conditions for fluorescent imaging in the methods

Author’s Response: The imaging methodology and scope details have been added to Method section.
Second decision letter

MS ID#: JOCES/2020/253757

MS TITLE: Human microsporidian pathogen Encephalitozoon intestinalis impinges on enterocyte membrane trafficking and signaling

AUTHORS: Juan Flores, Peter M Takvorian, Louis M Weiss, Ann Cali, and Nan Gao

ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks. As you will see, one reviewer suggested some very minor changes that could be made to the final manuscript further improve clarity.

Reviewer 1

Advance summary and potential significance to field

See my previous summary

Comments for the author

The authors have addressed all of my concerns and I now enthusiastically support the publication of the article.

Reviewer 2

Advance summary and potential significance to field

this is a revision, see previous review

Comments for the author

We thank the authors for their thoughtful and thorough response to our comments, which have all been well addressed.

A few minor comments that would be helpful to address prior to publication:

1) Figure 3K: Could the authors please note the number of biological replicates from which the quantification is made?

   Figure 5i: While the figure legend states a statistical analysis, we could not see any quantification shown in the figure. It seems this may have mistakenly not been inserted; please add in to the figure.

2) Figure 3E: Based on the current quantification/classification of mitochondria it appears that a vast majority of mitochondria within the control cells appear to not have normal cristae (blue). If the authors consider the blue and orange to both be normal, then perhaps merging these, or clarifying that the orange is also considered normal would be helpful? This was something that was still confusing to us.

3) Thank you for providing a schematic to explain the quantification of nuclear indentation in 4E. Could the schematic be annotated to indicate which vertex/line is actually being measured?

Author explanation: Height of the bottom of the concave portion of the nucleus was measured from the beginning of the host nuclear-PV interface

NOTE: We have removed a figure provided by the referee.