Author's response to reviews

Title: HIV-1 Tat Protein Alter the Tight Junction Integrity and Function of Retinal Pigment Epithelium: an in vitro Study

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Author's response to reviews: see over
Dear Dr. Rikki Graham

Thank you for your message concerning our manuscript entitled "HIV-1 Tat Protein Alter the Tight Junction Integrity and Function of Retinal Pigment Epithelium: an in vitro Study" (BMC Infectious Diseases Ms. No.: 1375774087181044). We also appreciate the reviewers’ kind comments on our manuscript and valuable suggestions to improve it. We fully dealt with the criticisms in the revised manuscript and underlined all the changes.

I specify changes made in the manuscript below, replying point-by-point to the reviewers’ comments. We rewrote the result and discussion section, so we did not specify the number of page or line where we made change. Sorry for the inconvenient to your work.

Referee 1: 1804960206184263_comment

Major comments

1) Although the observation that Tat exposure affects barrier function is interesting, this is a descriptive paper which also requires substantial revision in the presentation of the data as well as intensive English editing. Perhaps the authors could select one of the proteins affected and determine the mechanism by which Tat alter its level of expression, protein stability.

Answer: Thank you for your helpful suggestion. We have investigated the activation of ERK and NF-κB to determine the mechanism by which HIV-1 Tat alters the expression of TJ, and rewritten the most part of result and discussion. We have asked an editing company to modify our manuscript and revised it carefully by ourselves.

2) Consistent with all the other figures, also figures 5 and 6 should include Hi-Tat as an additional control.

Answer: We have added the Hi-Tat for control in mRNA and protein expression part.

Minor comments

Figure 2 can be combined with figure 3.
Answer: Thank you for your helpful suggestion. I have combined two figures as figure2a and 2b. Because the units of horizontal-axis in two figures were different, I think it would be hard to see it clearly in one picture (as shown below), I just combined them as two parts of figure2.

Referee 2: 8292284391860528_comment

Major compulsory revisions:

1) Tat protein is analyzed only at 100 nM concentration. The biological effects of Tat are tightly linked to its concentration. It is known that different concentrations of Tat may induce opposite biological effects in several cell models. It is basic that the Authors assay their cellular model with scalar concentration of Tat (0.1-1000 ng/ml) in order to find out whether the Tat has the same effects under different quantitative conditions.

Answer: Thank you for your helpful suggestion. In our experiments, we also found that HIV-1 Tat protein could induce different biological effects. In a low concentration (<100 nM), it mainly affect the phagocytosis and secretion of RPE, and has a slight effects on barrier function. If the concentration more than 200 nM, it will induce apoptotic cell death. 100 nM has also frequently been used in previous in vitro studies (Price, et al. 2005; Andras, et al. 2003). So we just wrote 100 nM in this paper.
2) The experimental controls were limited to heat-inactivated Tat. It is pivotal to treat D407 cells with scalar concentrations of at least another HIV protein, such as p24 gag protein to rule out the possibility that all the HIV protein may interfere with D407 tight junctions.

**Answer:** Our team is keeping on the research about the effects of HIV protein on RPE for couple of years. We also performed the effects of another HIV protein, gp120, on RPE. Although it also could induce the changes in TJs expression, the pattern of changes was different from HIV-1 Tat significantly (unpublished data). We didn’t mention it in this manuscript.

3) Tat effect was measured 24-72 hours after treatment. It is important to show the data of cell viability for the same time interval in order to wipe away any doubt regarding the survival of cells.

**Answer:** Thank you for your helpful suggestion. These data have been added in the results section.

4) In the text and in Figure 5, I have not noticed the exact entity of Claudins 1,3,4 mRNA decrease. Please add this data. Moreover the Tat-related mRNA and protein variation of Claudins is relatively low. The Authors must discuss this aspect.

**Answer:** Thank you for your helpful suggestion. The Tat-related mRNA and protein variation of Claudins is relatively low, we also found the decreases in expression of ZO-1 in another experiment (unpublished data). So we cannot exclude the possibility that other junctional proteins are also modulated by Tat and contribute to the observed effects on barrier function. The relationship TJs and the oBRB during HIV infection still need to be elucidated. These have been added in the discussion section.

5) The mRNA and eventually WB protein analysis must be performed until 72 hours of treatment at all scalar concentration of Tat.

**Answer:** We have performed the changes in expression of TJs at 48, and 72 hours, but they did not show significant difference between 24 hours, in accordance with result of TER. So we did not write it into this manuscript.

6) The results section must be rewritten melting several paragraphs because these paragraphs are focused on methods. This approach actually leads to repetition of
several parts and aspects that had already been described in the materials and methods section.

7) The discussion must be reduced. It is too long and dispersive.

Answer: We have rewritten the result and discussion section as you suggested.

In this paper, it is not proposed any experiment to study the mechanism(s) inducing the alteration of tight junctions. In the last lines of discussion section, the Authors cited that oxidative stress and ERK 1/2 are generally involved in the alterations of tight junctions. This aspect must be investigated because this paper is too descriptive and the analysis of mechanism(s) involved is fundamental. Moreover, in some cell systems, Tat strongly affect the proteins specifically involved in the regulation of oxidative stress (see the papers by Flores) and then the Authors can strongly improve their paper through this kind of analysis.

Answer: Thank you for your helpful suggestion. We have investigated the activation of ERK and NF-κB to determine the mechanism by which HIV-1 Tat alters the expression of TJs.

Discretionary revisions

The NIH reagent program has also Tat fragments. It would be important to analyze which fragment is involved in the alteration of tight junctions.

Answer: Thank you for your helpful suggestion. We’ll perform such experiment in the near further.

Referee 3: 1183884743187488_comment

1) 1. Is the question posed by the authors well defined? The authors stated the study objective in the “background” section of the abstract. There should also state the study rational here.

Answer: Thank you for your helpful suggestion. We have changed the background.

3) The data are sound, but weak and incomplete. This study will be strengthened by additional experiments looking into the mechanisms of Tat-induced dysfunction of brain-retinal barrier. In addition, the authors should explain their reasons for selection the TJs studied (occludin, claudins 1-4). Are other TJs proteins (ZO-1, ZO-2)
expressed in those cells? If yes, are they affected by Tat treatment? Do they play a role in Tat-induced dysfunction of brain-retinal barrier?

**Answer:** Thank you for your helpful suggestion. We have investigated the activation of ERK and NF-κB to determine the mechanism by which HIV-1 Tat alters the expression of TJs. We also found the decreases in expression of ZO-1 in another experiment. The other junctional proteins are also modulated by Tat and may contribute to the observed effects on barrier function. We will confirm it in the further study. Here we only show the changes in claudins.

5) The discussion is too long and could be shorten: e.g: Page 13, lines 2-6 is further description of the methods and should not be in the discussion. Also, some background literature can be move to the “Introduction – Background “ section, and only the study findings discussed in the Discussion section.

**Answer:** We have rewritten the result and discussion section as you suggested.

6) Limitations of the work are not stated. Example, these studies were performed in a cell line and may not necessary be extrapolated to human cells or in vivo situation. Confirming the finding using human primary retinal epithelial cells, and/or retinal autopsy tissues from infected and non-infected patients will add significant strength to this study.

**Answer:** Thank you for your helpful suggestion. We will perform your suggestion in the near future. We also added in conclusion section.

9) **Authors should do spell check and correct the mistakes mentioned below**

**Answer:** Thank you for your careful review. I have revised mistake you mentioned and checked for symbols and units.

1. Abstract. Background: “To determine the effects of HIV-1 Tat proteins on the barrier function and tight-junction protein expression of retinal pigment epithelial cell(RPE)” This appears to be the study objective or aim, not the background.

Conclusions: “ These maybe contribute to the pathogenesis of HIV-related ophthalmopathy.” Should be. “may contribute to…”

2. Page 4, line 6 “….TER and permeability…”, not “perimeability” Is it “fluorescence sodium” or “fluorescence sodium”? 
3. Reagent. Please give the source and molecular weight of fluorescence sodium.

4. Cell viability assay. Please check for symbols and units. Example, 150 ml DMSO is likely inappropriate and is probably 150-ml. There are several examples in the manuscript. This should be checked before and after uploading the manuscript, as the uploading process sometimes change / distort the symbols.

5. Page 5. Measurement of TER. Minor comments, there should be a space between number and units (e.g: 0.4mm; 50 ml, 0.6 cm2).

Page 6, 3rd and 4th lines. Should “400ul and 600ul” be “400 ml and 600 ml”? Line 9…”TER was measured by an epithelial voltohmeter…” should be “…TER was measured with an epithelial voltohmeter…”

Line 11. “…day10…” should have a space between day and 10. Page 7. Western blot analysis. Is it “…200 ml of ice-cold lysis buffer…” or “…200 ml of ice-cold lysis buffer…”?

Page 8, line 5…” (1:500).#-actin…” Please put a space between “(1:500)” and “#-actin”.

Page 8: Immunofluorescence microscopy.

Lines 1 and 2. Please put a space between “D407” and “cells”; “100” and “nM”.

Lines 7 and 8. “anti-occludin (10 mg/ml), anti-claudin-1 (2 mg/ml), anti-claudin-2 (4 mg/ml), anti-claudin-3 (4 mg/ml), anti-claudin-4 (4 mg/ml)”. Please check concentrations units for accuracy.

Page 9. TER analysis

“Because the TER appeared to be somewhat affected by the serum, so we reduce the serum concentration…” Please check / correct the sentence structure. Starting the sentence with “Because”, “so” is not appropriate here. “…for1 week…” need a space between “for” and “1”.

Page 11. Western blot analysis. Insert spaces to separate words where appropriate.

Answer: We have changed them. “400ul and 600ul” should be 400µl and 600µl.

Discussion:

1. Too long. Do not re-describe methods here, and only discuss the study findings.
2. Check and correct sentence structures. Examples a. page 14 “…Certain claudins express high tissue- and cell-type specificities….” b. Page 15, 2nd paragraph… “…Furthermore, it can selective increase the paracellular conductivity…”

3. How can expression of claudin-2 disrupt and decrease tightness of the epithelial barrier? Has similar observations been made in other studies or in other cell types?

Answer: We have rewritten the discussion section. Here is an article indicated that the addition of claudin-2 markedly decreased the tightness of individual claudin-1/4–based TJ strands, leading to the speculation that the combination and mixing ratios of claudin species determine the barrier properties of individual TJ strands(Furuse, et al. 2001).

We appreciate all the comments and suggestions from the reviewers. Fortunately, we are keeping on researching the effects of HIV protein on RPE, now we can add our latest result into this manuscript. We hope that the changes made to the manuscript meet with the reviewers’ satisfaction. Thank you for your kind consideration.

Sincerely,
Zhenping Zhang

References

