Author’s response to reviews

Title: Reduced transient receptor potential vanilloid 2 expression in alveolar macrophages causes COPD in mice through impaired phagocytic activity

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Author’s response to reviews:

Response to Reviewer #1

We would like to thank the reviewer #1 for providing us with valuable comments that has allowed us to improve the quality of our manuscript.

Comment 1. Figure 1: The Immunofluorescence images are not clear. Higher magnification images with counterstain (DAPI or Hoechst) are needed.
Response:

According to your suggestion, we added the image of DAPI in Figure 1, and changed the pictures to the higher resolution images. We hope the imaging quality will not be affected during the conversion to PDF.

Comment 2. Figure 2: Please indicate molecular weight. Please provide a full length gel showing the other bands (if any) that were present on the gel. Also, the B-actin loading control is not uniform, suggesting that less protein may have been loaded in the 24h lane column.

Response:

We appreciate the valuable comments. As pointed out, β-actin levels after 24 hr-exposure to 10% CSE were decreased. This is probably due to the cellular damage. Therefore, TRPV2 levels were standardized by β-actin levels, and TRPV2/β-actin ration at t=0 was set at 1.0. Furthermore, we added the molecular weight in Western blots (Figure 2A, Figure 4A, Figure 4B, Supplementary Figure 1A, and Supplementary Figure 2). In addition, according to the suggestion, Western blots were shown in full length gels below.

Figure 2A

TRPV2 β-actin

Figure 4A

TRPV2 β-actin

Figure 4B

TRPV2 β-actin
Comment 3. Figure 1 and Figure 4: Not all F4/80 cells seem to also express TRPV2. What % of F4/80 cells were TRPV2+. This can be done 2 ways: if the authors are able to perform flow cytometry, gating on TRPV2 followed by F4/80+/- would be useful. If not, counting of % positive cells by a blinded investigator on immunofluorescence should be done. Conversely, does TRPV2 stain cells that are F4/80 negative, and if so, what could these cell types be?

Response:

According to the comments, we counted the TRPV2 positive cells among F4/80 positive cells by a blinded investigator because we could not perform flow cytometry at this point. While 57.5±2.9% of F4/80-positive cells were TRPV2+ in vehicle group, only 37.2±3.4% of F4/80-positive cells were TRPV2+ in 10% CSE group (page 12, line 7). We noted that alveolar epithelial cells were positive for TRPV2 but negative for F4/80 in lung tissue from mice.

Comment 4. Can FITC-dextran internalization be imaged? It would be more compelling to show images of internalized FITC-dextran in WT and siTRPV2 or CS-exposed cells to show that the amount of phagocytosed dextran is lower.

Response:

We appreciate your valuable comments. According to your suggestion, we show the images of internalized FITC-dextran in MH-S cells with or without CSE. MH-S cells were incubated with 0.35 mg/ml FITC-dextran at 37°C for 6 hr. Then, cells were washed three times with PBS. Uptake of FITC-dextran by macrophages was assessed by a fluorescence microscope. As shown in pictures below, even in the absence of FITC-dextran, MH-S cells gave weak but discernible fluorescence. In the presence of FITC-dextran, while the fluorescence in cytoplasm became stronger in vehicle-treated MH-cells, it failed to do so in 10% CSE-exposed MH-S cells.
Comment 5. TRPV4 levels: What were the % macrophages in the cell diffs of BAL performed in CSE mice. Were the cells from the BAL sorted prior to analysis. If not, it is clear that the reduction of TRPV2 levels could be attributed to decreased macrophage TRPV2 levels since other cell types are present in the BAL. If unsorted BAL cells were used, the authors will need to state this in the discussion and couch the discussion with appropriate caveats re: the BAL data.

Response:

We appreciate your valuable comments. We isolated macrophages from BAL cells by plating the cells on the culture dishes which preferentially allow macrophages to adhere. We found that approximately 95% of BAL cells were adherent to the plates. Because we used unsorted BAL cells in our study, we stated the caveats of this method in Discussion section as shown below (page 16, line 5).

We prepared macrophages from BAL cells by plating the BAL cells on culture dishes over-night and then by removing the non-adherent cells. We should be aware that this method may have allowed some non-macrophages to be included in adherent cells. Thus, a decrease in TRPV2 protein in macrophages in BAL cells may be, at least in part, due to a decrease in the number of macrophages in BAL cells rather than the reduced expression of TRPV2 in macrophages.

Comment 6. Discussion: "evidence indicating that development of smoke-induced COPD is partly attributed to reduced phagocytosis". This claim seems premature. First, do TRPV2-/- mice develop worsened smoke-induced OLD (as assessed by histology scores of emphysema, pulmonary function testing). Ideally, this should be done in mice that are only deficient for TRPV2 in macrophages, but at a minimum, data on COPD development/severity in TRPV2-/- mice are needed to substantiate the claim that this channel plays a role in COPD pathobiology.

Response:

We appreciate the valuable comments. We agree that from the data presented here, it is premature to state that development of smoke-induced COPD is partly attributed to reduced phagocytosis. As the reviewer suggested, experiments to investigate the effects of smoking on lung structure and function in TRPV2-/- mice were required to substantiate our hypothesis. Accordingly, our claim should be toned down to just mention that we provided several lines of
possibility, instead of evidence, indicating the development of smoke-induced COPD is partly attributed to reduced phagocytosis (page 14, line 21).

Response to Reviewer #2

We would like to thank the reviewer #2 for providing us with valuable comments that has allowed us to improve the quality of our manuscript.

Comment 1. The major results (i.e. increased alveolar space enlargement in TRPV2 knockout mice) should be presented in a figure pertaining to the original manuscript rather than a supplementary figure.

Response:

According to the suggestion, we moved supplementary Figure 3 to Figure 6 that shows alveolar space enlargement in TRPV2 knockout mice and we added the some sentences in Results section to explain the findings (page 14, line 9). Moreover, original Figure 6 was moved to Figure 5, original Figure 5 moved to Figure 4 and original Figure 4 was moved to Supplementary Figure 4.

Comment 2. Please provide more details for the morphometry analysis in the Results section. Did you use an analytical software to measure the airspace enlargement? Were the airways and blood vessels excluded from the analysis?

Response:

According to the reviewer’s suggestion, we added the sentences in Methods section as shown below (page 10, line 1).

Lm is the average distance between alveolar walls and proportional to the amount of pulmonary emphysema. 20 randomly selected representative images were captured from each slide using a motorized OLYMPUS microscope per lung specimen for each mouse. Lm was manually counted from images taken using a winROOF2013. All measurements were performed by a single blinded investigator. The lengths of all the portions on all lines were summed and divided by the total number of alveolar airspace. Airway and vascular structures were excluded from the analysis.
Comment 3. Figure 1: Please give a plot with mean numbers (+/- SEM) to quantify TRPV2+ F4/80+ double positive cells over F4/80+ cells with the different treatments.

Response:

We appreciate the valuable comments. While 57.5+2.9 % of F4/80-positive cells were TRPV2+ in vehicle group, 37.2+3.4 % of F4/80-positive cells were TRPV2+ in 10% CSE group (page 12, line 7).

Comment 4. Figure 2: Are cells starting to die after 24 hours of 10% CSE? Has a viability assay been performed? The B-actin band intensity is decreased after 24 hours.

Response:

The referee raises an important issue. As pointed out, exposure of macrophages to 10% CSE for 24 hr caused cellular damage as detected by TUNEL assays. To demonstrate the decrease in TRPV2 expression in 24 hr column was not due to the nonspecific toxic effects of 10% CSE, we expressed the expression levels of TRPV2 relative to β-actin expression levels.

Comment 5. Figure 2: the legend does not correspond to the data shown.

Response:

We are very sorry about the careless mistake. We corrected it (legend of Figure 2).

Comment 6. Figure 3: How can luminescence be detected in the absence of FITC-dextran? A better representation would be to compare the intensity in vehicle versus CSE-treated samples, which would make the description of the data in the manuscript less confusing. Also, the legend does not correspond to the data shown.

Response:

We appreciate this important comment. According to the suggestions, in Supplementary Figure 3 in the revised manuscript, we showed the data of the luminescence intensity of cell lysates prepared from MH-S cells exposed to either vehicle or 10% CSE for 24 hr in the absence of FITC-dextran. Despite the absence of FITC-dextran, luminescence intensity was increased after
24 hr exposure to either vehicle or 10%CSE. Such an increase, which is probably due to augmented photoluminescence of cellular structures (autofluorescence), was more pronounced in 10% CSE-exposed cells compared with vehicle-exposed cells. In the presence of FITC-dextran, an increase in luminescence intensity was less in 10%CSE-exposed cells compared with vehicle-exposed cells at any time points (6, 12, 24 hr).

We corrected the legend for Figure 3 appropriately (legend of Figure 3).

Comment 7. Figure 4: Not sure what Figure 4 is supposed to show, TRPV2 has already been shown to be expressed in macrophages. This figure could certainly be moved to Supplementary Material. A quantification and comparison with air-exposed mouse lung staining would give more sense to this Figure. Are the samples from 2 or 6 months CS exposure?

Response:

We appreciate the valuable comments. Figure 4 in the original manuscript was meant to show that TRPV2 is expressed in alveolar macrophages (F4/80-positive cells) in mice using lung specimens from non-smoked mice. According to the comment, we moved Figure 4 to supplementary Figure 4.

Comment 8. Figure 5: The description in the body of the manuscript does not match the data shown in the Figure.

Response:

We are very sorry about the mistake. We corrected the description (page 13, line 13). Original Figure 5 was moved to Figure 4 in the revised manuscript.

Comment 9. Figure 6: The comments for Fig 3 can be applied here as well. Fig 6B is not that convincing and should mention TRPV2KO mice in the legend.

Response:

We are very sorry about the mistake. We added the sentence in the legend about TRPV2 KO mice, and corrected the comments for Figure 3. Furthermore, we moved original Figure 4 to Supplementary Figure 4, and original Figure 6 to Figure5.
Comment 10. Supplementary Figure 3: Panels A and C are interesting but somehow redundant with Panels B and D. Consider showing pictures of non-smoked lungs and the same time-course of CS exposure in WT animals. This Figure should be part of the main manuscript.

Response:

We are very sorry about the mistake. Data description of Supplementary Figure 3 was wrong. Panels B and D compared the alveolar space enlargement before and after 2-month smoking between 2-month-old WT and TRPV2KO mice. We corrected the figure and moved to Figure 6 (Original Figure 6 was moved to supplementary Figure 4).

Comment 11. Discussion: TRPV2 mRNA expression is discussed but no data are showing mRNA results.

Response:

We are very sorry about the mistake. We added the sentence in Method section (page 11, line 11), and Results section (page 12, line 11).

Comment 12. The authors state that "TRPV2 may provide a therapeutic target for COPD" but is it clinically druggable? Please discuss.

Response:

The referee raises an important issue. If we can inhibit the reduction of cigarette-induced TRPV2 expression by medications, TRPV2 might be druggable target for COPD. For example, probenecid, the prototypical uricosuric agent which acts as TRPV2 agonist (Transient receptor potential V2 expressed in sensory neurons is activated by probenecid. Bang S, et al. Neurosci Lett. 2007;425:120-5) may have potential to ameliorate COPD. We added following sentences in Discussion section (page 17, line 11).

Our finding that TRPV2-mediated phagocytosis is impaired in smoke-exposure mice suggests that TRPV2 agonists may have potential to ameliorate cigarette-induced COPD in humans. It is intriguing to speculate that probenecid, the prototypical uricosuric agent which has been reported to activate TRPV2 (Bang S, et al. Neurosci Lett. 2007;425:120-5), may have therapeutic potential for COPD. This possibility warrants thorough investigation in future studies.
Comment 13. The source, rather than the generation of TRPV2KO mice should be described before the Mice paragraph in the Methods section. It would allow the removal of the sentence "contents of the intervention was similar to wild-type mice" which is difficult to interpret as stated.

Response:

We changed the order between TRPV2KO mice and Mice in the Methods section according to the reviewer’s suggestion. And we deleted "contents of the intervention was similar to wild-type mice" (page 8, line 17).

Comment 14. For all the Western blot presented, a molecular weight should be mentioned for each assay.

Response:

According to your suggestion, we added the molecular weight in Western blots (Figure 2A, Figure 4A, Figure 4B, Supplementary Figure 1A, and Supplementary Figure 2).

Comment 15. Several typos can be found throughout the manuscript and need your attention:

- Are the BAL cells really spun at 3,000 g?
- A red secondary antibody was used for the immunofluorescence of TRPV2, please describe in the Methods section. Is the same truncated TRPV2 antibody being used for immunofluorescence, immunohistochemistry and Western blot?
- "All mice were euthanized by sevoflurane before tissue harvest"
- Remove "respectively" in the first paragraph of the Results section
- "Figure 1. Changes in TRPV2 expression in MH-S cells by immunofluorescence".

Response:

We are very sorry about the mistake. We changed the sentence according to your suggestion. And 3,000g was 3,000rpm actually.