Peer Review File

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Reviewer A

Comment 1: The authors were able to show that ZO1 and VECadherin are down regulated after LPS treatment using Western blot. Can they also verify that using standard immunofluorescent stainings of the endothelial monolayer.

Reply 1: According to your professional comment and the comment 4 of reviewer B, we tried to verify the expression of ZO1 and VE-Cadherin by using immunofluorescent staining of the endothelial monolayer. Unfortunately, we used several primary antibodies against VE-cadherin but failed to obtain a specific fluorescent signal for VE-Cadherin in endothelial cells. Therefore, we only presented the immunofluorescent staining images of ZO1 in the revised manuscript. As shown in the revised Figure 2 and Figure 5, in consistent with the western blot results, immunofluorescence staining showed that autophagy inhibitor 3-MA (Fig.2B), siRNA-mediated Atg5 knockdown (Fig.2F), FOXO1 inhibitor AS1842856 (Fig.5B), and siRNA-mediated FOXO1 knockdown (Fig.5F) significantly blocked the LPS-induced loss of ZO-1 in the plasma membrane of MLVECs.

We have added these findings in the section of "Result" in the revised manuscript (see Page 12, line 11,21; Page 14, line 17, Page 15,line 2).

Comment 2: Does the treatment with LPS induce cell death. The authors should exclude that in order to state that it LPS induces autophagy.

Reply 2: Thanks a lot for your professional comment and the comment 2 of reviewer B. Previous studies have link FOXO1 to apoptosis in endothelial cells *(Zhang H, Ge S, He K, Zhao X, Wu Y, Shao Y, Wu X. FoxO1 inhibits autophagosome-lysosome fusion leading to endothelial autophagic-apoptosis in diabetes. Cardiovasc Res. 2019; 115(14): 2008-2020. Xie Y, Li X, Ge J. Cyclophilin A-FoxO1 signaling pathway in endothelial cell apoptosis. Cell Signal. 2019; 61: 57-65.)*. In fact, another work in our group detected the effect of LPS and FOXO1 inhibitor on endothelial cell apoptosis. As shown in the figure below, LPS treatment induces apoptosis in primary cultured MLVECs, as evidenced by increased TUNEL-positive cells and caspase-3 activity. In addition, FOXO1 inhibitor AS1842856 pretreatment significantly reversed LPS-induced apoptosis in MLVECs. The mechanisms involved in LPS-induced endothelial cell apoptosis is the key work of another student in our group, therefore we didn't add this figure in

the revised manuscript. Instead, we have added a description of limitation in the revised manuscript as following: “Certain limitations should be considered regarding this study. First, FOXO1 has the additional non-autophagy related cellular effects on endothelial cell injury and is also involved in endothelial cell apoptosis. The benefits of FOXO1 inhibition illustrated in this study might not be entirely attributed to its inhibitive effect on autophagy.”. (see Page 18, line 18-21; Page 19, line 1-4).

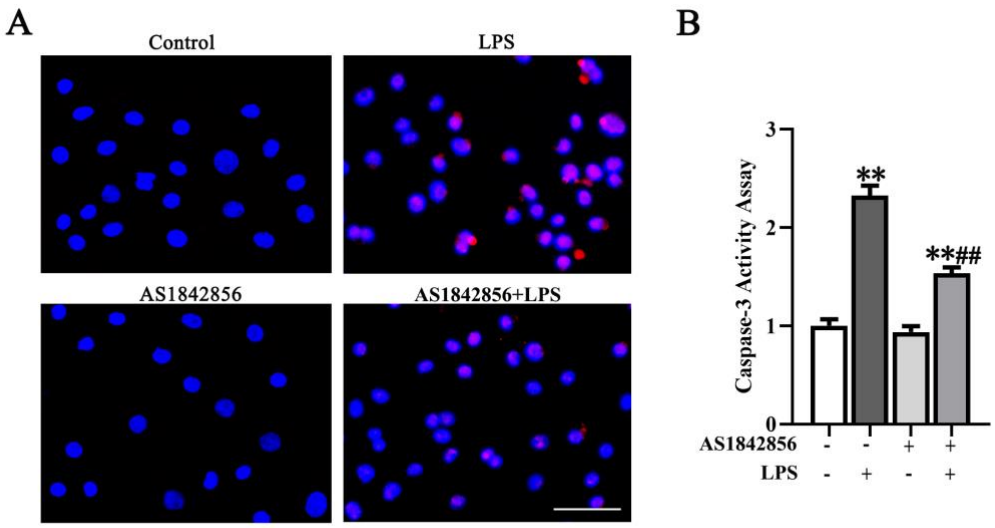

Figure. FOXO1 inhibition reversed LPS-induced apoptosis in pulmonary vascular endothelial cells. MLVECs were treated with LPS (1,000 ng/ml) with or without FOXO1 inhibitor AS1842856 (10 μM) for 24 hours. A, showed representative TUNEL-stained (red) cells. Nuclei were counterstained with DAPI (blue). B, showed caspase 3 activities. Data are presented as the mean ± SEM (n=4). ** $p < 0.01$ vs control group. ## $p< 0.01$ vs LPS group.

Comment 3: The authors assess only FOXO1 protein levels. But FOXO1 is a transcription factor that is frequently shuttling between the nucleus, where it is active, and the cytoplasm where it is inactive. Therefore, the authors should check for FOXO1 phosphorylation as well as for FOXO1 sub cellular localization, in order to get more information, whether the increased FOXO1 levels are active FOXO1 in the nucleus or not.

Reply 3: Thank you for your professional comment. We agree that we should check for FOXO1 phosphorylation as well as for FOXO1 sub-cellular localization, in order to get more information. As shown in the revised Figure 3, LPS treatment caused significant increases in the protein levels of phosphorylated-FOXO1 in a dose-dependent manner over a 24-hour

incubation period. Immunofluorescence staining showed that LPS treatment led to profound FOXO1 nuclear translocation in MLVECs (Supplemental Figure 1). As shown in the revised Figure 4A and Supplemental Figure 1, AS1842856 pretreatment significantly decreased LPS-induced FOXO1 phosphorylation and FOXO1 nuclear translocation in MLVECs. We also examined the effect of AS1842856 and/or LPS on phosphorylated-FOXO1 levels in lung tissues. As shown in the revised Figure 6A, administration of AS1842856 significantly decreased LPS-induced upregulation of phosphorylated-FOXO1.

We have added these findings in the section of "Result" in the revised manuscript (see Page 13, line 11; Page 13, line 18; Page 15, line 10).

Comment 4: The authors use a FOXO1 inhibitor. Here is the weakest point of the manuscript in my opinion, because the authors do not confirm that the drug also works. They show that LPS increases FOXO1 protein levels, but they do not show whether FOXO1 inhibitor actually decreases the protein level or whether it changes FOXO1 sub cellular localization or how does it work? Additionally, they could knockdown FOXO1 and see whether this recapitulates the effect they see, in order to corroborate their data.

Reply 4: Thank you for your professional comment.

1. FOXO1 inhibitor AS1842856 is known to predominantly suppresses FOXO1-mediated transactivation by directly binding to FOXO1 (Nagashima T, et al. Discovery of novel forkhead box O1 inhibitors for treating type 2 diabetes: improvement of fasting glycemia in diabetic db/db mice. Mol Pharmacol. 2010 Nov;78(5):961-70.). According to your comment, we examined the effect of AS1842856 and LPS on FOXO1 phosphorylation as well as for FOXO1 sub-cellular localization in endothelial cells. As shown in Figure 4A and Supplemental Figure 1, AS1842856 pretreatment significantly decreased LPS-induced FOXO1 phosphorylation and FOXO1 nuclear translocation in MLVECs. We also examined the effect of AS1842856 and/or LPS on phosphorylated-FOXO1 levels in lung tissues. As shown in Figure 6A, administration of AS1842856 significantly decreased LPS-induced upregulation of phosphorylated-FOXO1.
2. According to your comment, we then investigated whether knockdown of FOXO1 affected LPS-induced autophagy and pulmonary endothelial injury in the revised manuscript. As shown in Figure 4D, siRNA-mediated FOXO1 knockdown not only led to a significant decrease of FOXO1 expression in MLVECs, but also blocked the LPS-induced upregulation of FOXO1 and phosphorylated-FOXO1. FOXO1 knockdown blocked the LPS-induced increases in protein levels of LC3-II and ULK1 (Figure 4D. As shown in Figure 4E&F, FOXO1 knockdown

significantly reduced the number of both autophagosomes and autolysosomes in LPS-treated MLVECs. These findings indicated that FOXO1 knockdown could suppress LPS-induced autophagy in pulmonary vascular endothelial cells. We then investigated whether FOXO1 knockdown affected LPS-induced pulmonary endothelial injury. As shown in Figure 5E, FOXO1 knockdown blocked the LPS-induced decreases in protein levels of VE-cadherin and ZO-1. Immunofluorescence staining showed that FOXO1 knockdown significantly reversed the LPS-induced loss of ZO-1 in MLVECs (Figure 5F&G). Furthermore, LPS-induced endothelial cell hyperpermeability was also significantly alleviated by FOXO1 knockdown (Figure 5H). Collectively, these results indicated that FOXO1 knockdown protected against LPS-induced pulmonary endothelial injury.

We have added these findings in the section of "Result" in the revised manuscript (see Page 13, line 18; Page 15, line 10; Page 14, line 8-11; Page 15, line 3-5).

Reviewer B

Comment 1: My main concern lies on the specificity of the study regarding the effects on EC autophagy. The authors suggest autophagy is the cause of LPS-induced pulmonary EC injury. However, the autophagy inhibitor employed, 3MA, is a PI3K inhibitor capable of impacting other cellular pathways in addition to suppressing autophagy in ECs. Therefore, I suggest the authors perform the EC barrier function experiments (permeability and levels of junctional molecules) in a genetically deficient autophagy background, such as knocking down core autophagy genes (Atg5, Atg7) using siRNA or lentiviral approaches.

Reply 1: Thank you for your professional comment. We agree that the autophagy inhibitor 3MA is also a PI3K inhibitor capable of impacting other cellular pathways in addition to suppressing autophagy in endothelial cells. According to your comment, we then investigated whether knockdown of the core autophagy gene Atg5 affected LPS-induced pulmonary endothelial injury in the revised manuscript. As shown in the revised Figure 2E, siRNA-mediated Atg5 knockdown not only led to a significant decrease of Atg5 expression in MLVECs, but also blocked the LPS-induced upregulation of Atg5. Atg5 knockdown blocked

the LPS-induced decreases in protein levels of VE-cadherin and ZO-1 (Figure 2E). Immunofluorescence staining showed that Atg5 knockdown significantly reversed the LPS-induced loss of ZO-1 in MLVECs (Figure 2F&G). Furthermore, LPS-induced endothelial cell hyperpermeability was also significantly alleviated by Atg5 knockdown (Figure 2H). Collectively, these results indicated that autophagy inhibition could attenuate LPS-induced pulmonary endothelial injury.

We have added these findings in the section of "Result" in the revised manuscript (see Page 12, line 17-21; Page 13, line 1-3).

Comment 2: On the other hand, it is not clear whether FOXO1 inhibition is protecting against ALI through induction of autophagy. Lung ECs can exhibit enhance cell death in response to endotoxemia and autophagy might be upregulated as a pro-survival mechanism. Since FOXO1 has been linked to EC death pathways, as the authors point out (reference 35), exploring EC viability/apoptosis (activated Caspase 3,etc) in response to LPS and FOXO1 inhibition will be informative.

Reply 2: Thanks a lot for your professional comment and the comment 2 of reviewer A. Previous studies have link FOXO1 to apoptosis in endothelial cells *(Zhang H, Ge S, He K, Zhao X, Wu Y, Shao Y, Wu X. FoxO1 inhibits autophagosome-lysosome fusion leading to endothelial autophagic-apoptosis in diabetes. Cardiovasc Res. 2019; 115(14): 2008-2020. Xie Y, Li X, Ge J. Cyclophilin A-FoxO1 signaling pathway in endothelial cell apoptosis. Cell Signal. 2019; 61: 57-65.)*. In fact, another work in our group detected the effect of LPS and FOXO1 inhibitor on endothelial cell apoptosis. As shown in the figure below, LPS treatment induces apoptosis in primary cultured MLVECs, as evidenced by increased TUNEL-positive cells and caspase-3 activity. In addition, FOXO1 inhibitor AS1842856 pretreatment significantly reversed LPS-induced apoptosis in MLVECs. The mechanisms involved in LPS-induced endothelial cell apoptosis is the key work of another student in our group, therefore we didn't add this figure in the revised manuscript. Instead, we have added a description of limitation in the revised manuscript as following: "Certain limitations should be considered regarding this study. First, FOXO1 has the additional non-autophagy related cellular effects on endothelial cell injury and is also involved in endothelial cell apoptosis. The benefits of FOXO1 inhibition illustrated in

this study might not be entirely attributed to its inhibitive effect on autophagy.". (see Page 18, line 18-21; Page 19, line 1-4).

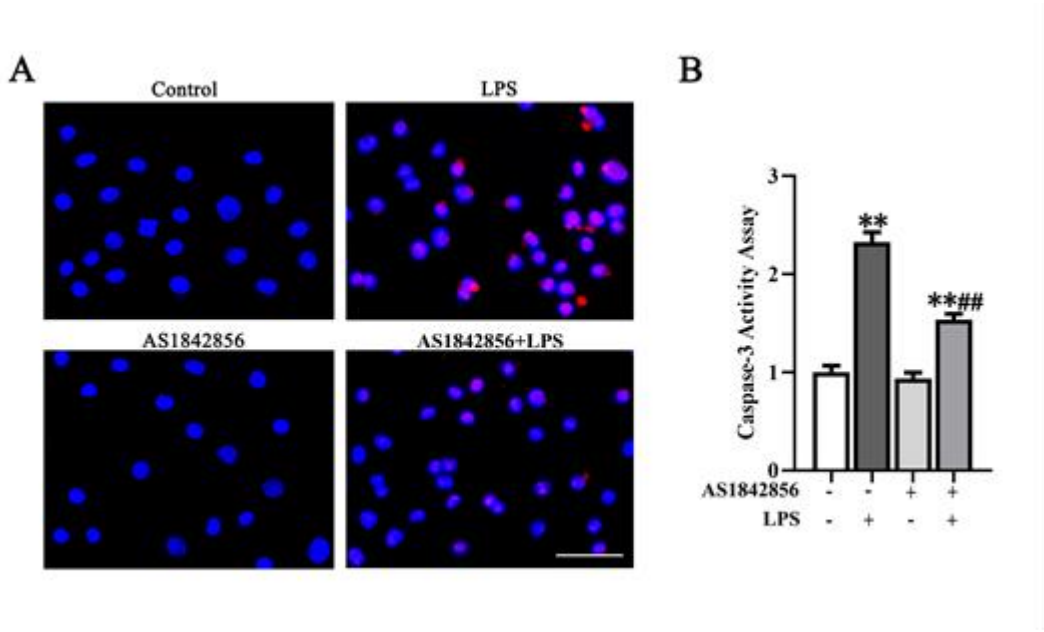

Figure. FOXO1 inhibition reversed LPS-induced apoptosis in pulmonary vascular endothelial cells. MLVECs were treated with LPS (1,000 ng/ml) with or without FOXO1 inhibitor AS1842856 (10 μM) for 24 hours. A, showed representative TUNEL-stained (red) cells. Nuclei were counterstained with DAPI (blue). B, showed caspase 3 activities. Data are presented as the mean ± SEM (n=4). ** $p < 0.01$ vs control group. ## $p< 0.01$ vs LPS group.

Comment 3: Likewise, the mechanism of FOXO1 inhibitor is not clearly explained in the text. Does this inhibitor regulate the expression levels of this transcription factor? Western blot analysis of FOXO1 levels in vitro and in vivo (Figures 4, 5 and 6) should be included.

Reply 3: Thank you for your professional comment. FOXO1 inhibitor AS1842856 is known to predominantly suppresses FOXO1-mediated transactivation by directly binding to FOXO1 *(Nagashima T, et al. Discovery of novel forkhead box O1 inhibitors for treating type 2 diabetes: improvement of fasting glycemia in diabetic db/db mice. Mol Pharmacol. 2010 Nov;78(5):961-70.)*. As mentioned by Reviewer A (Comment 3), FOXO1 is a transcription factor that is frequently shuttling between the nucleus, where it is active, and the cytoplasm

where it is inactive. According to this comment, we examined the effect of AS1842856 and LPS on FOXO1 phosphorylation as well as for FOXO1 sub-cellular localization in endothelial cells. As shown in Figure 3, LPS treatment caused significant increases in the protein levels of phosphorylated-FOXO1 in a dose-dependent manner over a 24-hour incubation period. Immunofluorescence staining showed that LPS treatment led to profound FOXO1 nuclear translocation in MLVECs (Supplemental Figure 1). As shown in Figure 4A and Supplemental Figure 1, AS1842856 pretreatment significantly decreased LPS-induced FOXO1 phosphorylation and FOXO1 nuclear translocation in MLVECs. We also examined the effect of AS1842856 and/or LPS on phosphorylated-FOXO1 levels in lung tissues. As shown in Figure 6A, administration of AS1842856 significantly decreased LPS-induced upregulation of phosphorylated-FOXO1.

We have added these findings in the section of "Result" in the revised manuscript (see Page 13, line 6-20).

Comment 4: Finally, it will strengthen the manuscript if the authors could provide data regarding the expression levels of junctional proteins (VE-cadherin and ZO-1) in LPS-treated MLECs by immunofluoresecence staining in addition to WB.

Reply 4: According to your professional comment and the comment 1 of reviewer A, we tried to verify the expression of ZO1 and VE-cadherin by using immunofluorescent staining of the endothelial monolayer. Unfortunately, we used several primary antibodies against VE-cadherin but failed to obtain a specific fluorescent signal for VE-cadherin in endothelial cells. Therefore, we only presented the immunofluorescent staining images of ZO1 in the revised manuscript. As shown in the revised Figure 2 and Figure 5, in consistent with the western blot results, immunofluorescence staining showed that autophagy inhibitor 3-MA (Fig.2B), siRNA-mediated Atg5 knockdown (Fig.2F), FOXO1 inhibitor AS1842856 (Fig.5B), and siRNA-mediated FOXO1 knockdown (Fig.5F) significantly blocked the LPS-induced loss of ZO-1 in the plasma membrane of MLVECs.

We have added these findings in the section of “Result” in the revised manuscript (see Page 12, line 11-13; Page 12, line 20-21; Page 15, line 1-3;).

Comment 5: Figure 1A : A key for the graph, indicating the different conditions for each bar, should be provided.

Reply 5: Thank you very much for your careful review. We feel really ashamed for our carelessness. We have provided the different conditions for each bar in all the revised figures (see the revised figures).

Comment 6: Figure 3: There is a space missing between “upregulated” and “in” in the figure title.

Reply 6: This error has been fixed in the revised manuscript (see Page 27, line 9).

Comment 7: Figure4B: The images should show cells with a single nucleus.

Reply 7: According to the comment 3 of reviewer C, we used a tandem fluorescence RFP-GFP-LC3 reporter system to monitor the autophagic flux in primary cultured MLVECs in the revised manuscript. MLVECs with a single nucleus have been shown in the images (see Page 11, line 17-20).

Reviewer C

Comment 1: Abstract - Background: Mention of FOXO1 is missing. Authors should explain and define this molecule/compound. Authors mention it in the result section but it belongs into the introduction/and method section.

Reply 1: According to your comment, we have explained and defined FOXO1 in the Abstract Background of the revised manuscript (see Page 5, line 20-23; Page 6, line 1-2).

Comment 2: - Methods: Grammar and style should be revised. There are many articles etc. missing, also the language style must be revised.

Reply 2: Thank you for your comment. With the help of the Language Editing Service via AME, our manuscript has been revised by two experienced scientists proficient in English. We have corrected the careless mistakes, spelling and grammar errors in the revised manuscript.

Comment 3: Results- The authors present an accumulation of autophagosomes and the upregulation of autophagy-related LC-3II and autophagy initiator ULK1 in LPS-induced ALI. These results demonstrate an accumulation of autophagic proteins, but do not allow for the description of autophagy induction as reduced autophagic flux could result in the same observations. Measurement of autophagic flux should be included in order to prove that the accumulation of autophagosomes is indeed due to autophagy induction.

- In this regard, the authors aim to inhibit autophagy in MLVECs by the application of 3-MA. The use of 3-MA comes with pitfalls the authors did not address. Importantly, 3-MA can induce autophagy if cell culture conditions are not optimal. Therefore, autophagic flux should be evaluated or a more robust method of autophagy inhibition should be chosen.
- The effects of FOXO1 on endothelial permeability and consecutive lung injury are convincingly demonstrated.

Reply 3: Thank you for your professional comment.

1. According to your comment, we used a tandem fluorescence RFP-GFP-LC3 reporter system to monitor the autophagic flux in primary cultured MLVECs in the revised manuscript. As shown in the revised Figure 1C and 1D, a marked increase in the number of both autophagosomes and autolysosomes was observed in MLVECs treated with LPS (1000 ng/ml) for 24 hours. Moreover, the number of both autophagosomes and autolysosomes in LPS-treated MLVECs were significantly reduced by FOXO1 inhibitor AS1842856 (Fig 4B and C) and siRNA-mediated FOXO1 knockdown (Fig 4E and F). These findings indicated that FOXO1 inhibition or knockdown could suppress LPS-

induced autophagy in pulmonary vascular endothelial cells.

2. As mentioned in this comment and the comment 1 of Reviewer B, we agree that 3MA is not a specific inhibitor for autophagy. 3-MA can induce autophagy if cell culture conditions are not optimal. 3-MA is also a PI3K inhibitor capable of impacting other cellular pathways in addition to suppressing autophagy in endothelial cells. Therefore, in the revised manuscript, we also investigated whether knockdown of the core autophagy gene Atg5 affected LPS-induced pulmonary endothelial injury. As shown in the revised Figure 2E, siRNA-mediated Atg5 knockdown not only led to a significant decrease of Atg5 expression in MLVECs, but also blocked the LPS-induced upregulation of Atg5. Atg5 knockdown blocked the LPS-induced decreases in protein levels of VE-cadherin and ZO-1 (Figure 2E). Immunofluorescence staining showed that Atg5 knockdown significantly reversed the LPS-induced loss of ZO-1 in MLVECs (Figure 2F&G). Furthermore, LPS-induced endothelial cell hyperpermeability was also significantly alleviated by Atg5 knockdown (Figure 2H). Collectively, these results indicated that autophagy inhibition could attenuate LPS-induced pulmonary endothelial injury.

   We have added these findings in the section of "Result" in the revised manuscript (Page 12, line 16-21; Page 13, line 1-3 Page 14, line 1-11).

Comment 4: Discussion- The limitations of the study in its current form regarding the evaluation of autophagy are not addressed.

Reply 4: According to your professional comment and the comments of reviewer B, we realized the limitations of our study regarding the evaluation of autophagy and the use of non-specific autophagy inhibitor 3-MA. Therefore, as we described in the aforementioned responses, we used a tandem fluorescence RFP-GFP-LC3 reporter system to monitor the autophagic flux, meanwhile investigated whether knockdown of the core autophagy gene ATG5 affected LPS-

induced pulmonary endothelial injury in the revised manuscript. Nevertheless, certain limitations should be considered regarding this study. First, FOXO1 has the additional non-autophagy related cellular effects on endothelial cell injury and is also involved in endothelial cell apoptosis. The benefits of FOXO1 inhibition illustrated in this study might not be entirely attributed to its inhibitive effect on autophagy. Second, this study used LPS-induced ALI model alone to investigate the contribution of FOXO1 to endothelial cell autophagy and dysfunction. Confirmation of the role of FOXO1 in other animal models such as ALI-induced by cecal ligation and puncture, acid aspiration or mechanical ventilation would further reinforce the choice of FOXO1 as a potential therapeutic target.

We have added the limitation of this study in the section of “Discussion” in the revised manuscript (Page 11, line 17-20; see Page 12, line 16-21; Page 13, line 1-3; Page 18, line 18-21; Page 19, line 1-4).