Author Response

Reviewer: 1

Comments to the Author

1. Was there any smooth muscle thickening? Appearance of perivascular myofibroblasts? If yes, the treatments had any effect on these features?

We appreciate the suggestion, through this we carry out new analyzes. For the assessment of myofibroblasts, we considered the analysis of actin. We also evaluated VEGF since myofibroblasts produce this endothelial vascular growth factor. This polypeptide growth factor is essential in the formation of new blood vessels. In addition, myofibroblasts produce endothelin 1, a potent vasoconstrictor, but also a factor that stimulates the formation of new myofibroblasts, so we also analyze endothelin to add that analysis.

Regarding the actin results, there was an increase in the OVA group (10.8±8.9) compared to the SAL group (1.9±1.2), $p<0.001$. The treated groups showed attenuation when compared to the OVA group, $p<0.001$. Treatment with the association of anti-IL17 to the Rho-kinase inhibitor also attenuated the expression of actin when compared to the OVA group ($p= 0.009$).

In the evaluation of endothelin 1, the OVA group (18.5±10.0) showed an increase in relation to the SAL group (1.2±1.6), $p<0.001$. The OVA-RHO inhibitor group showed a decrease compared to the OVA group ($p= 0.01$). The OVA-anti-IL17 group showed a decrease compared to the OVA group ($p<0.001$). Treatment with the association of anti-IL17 to the Rho-kinase inhibitor also attenuated the expression of actin when compared to the OVA group ($p<0.001$). The anti-IL17 group and the association of treatments potentiated attenuation when compared to the treatment group that used only Rho-kinase inhibitor ($p<0.001$).

These new analyzes were included in the results and in the discussion of the manuscript.

2. In the title, “in a chronic allergic pulmonary inflammation” should be rephrased to “induced by chronic allergic pulmonary inflammation”

We appreciate the suggestion, we changed the title to: “Effect of anti-IL17 and / or Rho-kinase inhibitor treatments on vascular remodeling induced by chronic allergic pulmonary inflammation”

3. Fig 1 Figure legends should be rewritten. Each figure should stand alone with concise clear message; the caption should be stated something like as “Anti-IL-17 or Rho-kinase inhibitor has no effect on angiogenesis in ovalbumin-induced chronic lung inflammation in mice”. After caption, briefly describe the model and treatments in one or two sentences, number of mice/groups? which stats methods were used? data are shown as mean ± standard error mean? ROHi, Ova abbreviation?

Thanks for the comment. We rewrote the caption.

Figure 1. In OVA group there was an increase in angiogenesis compared to the saline group. The inhibitor of Rho-kinase and anti-IL17 have no effect on angiogenesis in chronic allergic pulmonary inflammation induced by ovalbumin in mice.

N: 8 animals per group. SAL: saline group, OVA: animals induced to chronic allergic inflammation by ovalbumin, OVA- RHO inhibitor: animals with induction of chronic allergic pulmonary inflammation and use of Rho-kinase inhibitor, OVA-anti-IL17: animals with induction of chronic allergic pulmonary inflammation and use anti-IL17, OVA- RHO inhibitor-anti-IL17: animals with induction of chronic allergic pulmonary inflammation and use of Rho-kinase inhibitor and anti-IL17. To determine the difference between groups and their statistical significance, we used the unidirectional analysis of variance (ANOVA) followed by the Holm-Sidak method for multiple comparisons. The data are presented as dot plots with standard deviations.

4. Fig 2 Figure legends should be re-written. See above comment. ROHi, Ova abbreviation?

Thanks for the comment. We rewrote the caption in the previous question. We also fixed the graphic legend related to OVA-RHOi (ovalbumin and Rho-kinase inhibitor) and OVA (ovalbumin) abbreviation.

5. Fig 3 Figure legends should be re-written. See above comment. Which staining? Representative image for how many mice? ROHi, Ova abbreviation?

Thanks for the comment. The image represents the experimental groups (8 animals per group). As we reported in the previous question, we corrected the legend referring to OVA-RHOi and OVA abbreviation.

6. Figures 2 and 3 should be combined

Thanks for the comment. We combined the figures as suggested.

7. Fig 4 Figure legends should be re-written. See above comment.

Thanks for the comment. The figure legends were rewritten according to the previous answer.

8. Fig 5 Figure legends should be re-written. See above comment. Which staining? Representative image for how many mice? ROHi, Ova abbreviation?

Thanks for the comment. The figure legends were rewritten according to the previous answer 6. The image represents the experimental groups (8 animals per group). We corrected the legend referring to OVA-RHOi and OVA abbreviation.

9. Figure 4 and 5 should be combined

Thanks for the comment. We combined the figures as suggested.

10. Fig 6 Figure legends should be re-written. See above comment.

Thanks for the comment. We rewrote the figure legends.

11. Fig 7 Figure legends should be re-written. See above comment. Which staining? Representative image for how many mice?

Thanks for the comment. We rewrote the figure legends. The image staining the IL-17. The image represents the experimental groups (8 animals per group).

12. Figures 6 and 7 should be combined

Thanks for the comment. We combined the figures 6 and 7 as suggested.

13. Fig 8 Figure legends should be re-written. See above comment.

Thanks for the comment. We rewrote the figure legends.

14. Fig 9 Figure legends should be rewritten. See above comment. Which staining? Representative image for how many mice?

Thanks for the comment. We rewrote the figure legends. The image staining the MMP-12, TIMP-1 and TGF-β. The images represents the experimental groups (8 animals per group).

15. Figures 8 and 9 should be combined

Thanks for the comment. We combined the figures 8 and 9 as suggested.

16. Fig 10 Figure legends should be re-written. See above comment.

Thanks for the comment. We rewrote the figure legends according to the following text.

17. In Table 1, treatments doses? duration?

We reformulated the table for the experimental groups and included an image for the protocol.

18. Page 19, lines 47-56, please include reference

Thanks for the comment. The references were included in the text.

19. Page 20, lines 45-51, please include reference

Thanks for the comment. The references were included in the text.

20. Page 21 Line 26-31 objective should be removed.

Thanks for the comment. The objective has been removed.

21. Page 21 Line 47 Please include age of the mice

Thanks for the comment. The animals were 6 weeks old, we included this information in the text.

22. Page 23 Line 22-23 please reworded “in the sequence described by”

Thanks for the comment. We rewrote this text.

23. Page 25-line 6 standard error mean?

Thanks for the comment. After considerations we modified the data to standard deviation.

24. Page 25 line 25 Please re-write title of the result section. E,g anti-IL17 and/or Rho-kinase inhibitor treatments has no effect on angiogenesis in Ova-induced experimental asthma

Thanks for the comment. We re-write title of the result section.

25. Page 25 Please re-write title of the result section. The title should be informative, and reflect the results

Thanks for the comment. We re-write title of the result section. “Anti-IL17 and/or Rho-kinase inhibitor treatments attenuated Rho-kinase expression in experimental asthma induced by ovalbumin”

26. Page 26 line 5 and 31 Please re-write title of the result sections as suggested above

Thanks for the comment. We re-write title of the result section. “Anti-IL17 and/or Rho-kinase inhibitor treatments reduced the oxidative stress response in experimental asthma induced by ovalbumin“ and “Anti-IL17 and/or Rho-kinase inhibitor treatments attenuated inflammatory markers in experimental asthma induced by ovalbumin“

27. Page 27 lines 3, 22, 42 Please re-write title of the result sections as suggested above

Thanks for the comment. We re-write title of the result section. “Anti-IL17 and/or Rho-kinase inhibitor treatments reduced extracellular matrix remodeling in experimental asthma induced by ovalbumin“ and “Anti-IL17 and/or Rho-kinase inhibitor treatments attenuated controlling mechanisms including the expression of transcriptors factors in experimental asthma induced by ovalbumin”

28. Page 28 line 5 please completely re-write the first paragraph of the discussion. Please briefly describe background, then mention knowledge gap, how did you addressed, what you found, importance of the findings in couple of sentences in the first paragraph of discussion. Next discuss one by one the findings…….

Thank you for the considerations. We rewrote the first paragraph of the discussion.

“Asthma is a chronic disorder that results in variable airflow limitation in the airways and is associated with airway hyperresponsiveness, airway inflammation, and tissue remodeling. There are studies that analyze the effect of treatment with anti-Il17 or Rho-kinase inhibitor in the airways and alveolar septa, but there are no studies to date that analyzed the effect of these treatments on the vascular responses. This study, in an asthma model with chronic allergic pulmonary inflammation, analyzed angiogenesis, the expression of ROCK1 and ROCK2, inflammatory cells, and remodeling markers, and the controlling mechanisms. The results of this study showed that when treated with anti-IL-17 and/or Rho-kinase inhibitor, not observe differences in angiogenesis, there is a reduction in the number of NF-κB, FOXP3, dendritic cells, STAT1, phospho-STAT1, ROCK1, and ROCK2 positive cells. There is also a decrease in the expression of the inflammatory markers, such as TNF-α, CD4+, CD8+, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, and IL-17 positive cells; remodeling markers, such as TIMP-1, MMP-9, MMP-12, TGF-β, and VEGF positive cells; and decorin, fibronectin, biglycan, and collagen

fibers. In addition, these treatments also reduced the expression of the oxidative stress markers iNOS and isoprostane."

29. Page 30-line 3 asthma models?

Thank you for the comment. These results were obtained analyzing in asthma models. We include this information in the text.

"In this study, we demonstrated the attenuation of CD4+ and CD8+ positive cells in the vessel walls of the animals treated with anti-IL-17 and/or Rho-kinase inhibitor. These results were similar to our recent studies analyzing airways and pulmonary parenchyma in model of chronic allergic pulmonary inflammation (14, 15)."

Reviewer: 2

Comments to the Author

General comments

1. It would be clearer to the readers if the authors would refer to their previous study as their own work. Eg. Page 29, Line 24 "A recent study by Santos et al…" should be changed to "Our recent study…"

Thanks for the suggestion we rewrote the way we refer to our work and more.

2. Similarly, the authors should reference studies by other authors when referring to "results (that) were similar to those found in the literature (Page 30, Line 5).

Thanks for the suggestion, we rewrote the form when we quote our work and others.

3. Could the authors comment on why only one technique was used to evaluate angiogenesis. Why were other markers of angiogenesis not quantified, ie. vWF, amphiregulin, or endothelin-1?

Thanks for the suggestion, we evaluated endothelin after consideration. In the evaluation of endothelin 1, the OVA group (18.5±10.0) showed an increase in relation to the SAL group (1.2±1.6). The OVA-RHO inhibitor group showed a decrease compared to the OVA group ($p=0.01$). The OVA-anti-IL17 group showed a decrease compared to the OVA group ($p<0.001$). Treatment with the association of anti-IL17 to the Rho-kinase inhibitor also attenuated the expression of actin when compared to the OVA group ($p<0.001$). The anti-IL17 group and the association of treatments potentiated attenuation when compared to the treatment group that used only Rho-kinase inhibitor ($p<0.001$).

These new analyzes were included in the results and in the discussion of the manuscript.

4. Could the authors comment on the length of time the mice were challenged with OVA? Other studies use much longer exposure times (several weeks) before culling for angiogenesis assessment.

The animals were challenged with ovalbumin on days 22, 24, 26 and 28 of the experimental protocol. Previous studies demonstrate that it is possible to evaluate angiogenesis with an experimental protocol in which animals receive ovalbumin solution during the period of 4-5 weeks (Adi S.D. et al., 2019; Zhang F. et al. 2017., Prado C.M. et al. 2011). We have included these points in the discussion of the manuscript.

Major Changes

1. The IHC is rather unclear. Please provide images with higher DPI.

Thanks for the comment. The images were corrected to 300-400 DPI.

2. Please provide quantification of vessel area.

We quantified the external perimeter of the vessels to quantify the vascular wall area. We measured the smooth muscle and the adventitious wall areas and the results were expressed in μm2. There was an increase in the area of the vascular wall of the OVA group compared to the Sal group ($p<0.001$). The treated groups were not different from both control and OVA animals.

Minor Changes

1. Please display graphs as dot plots and indicate that the full cohort of 8 mice is quantified for each measurement.

Thank you for the comment. The graphics display has been corrected.

2. Please provide standard deviations or interquartile range in graphs instead of standard error of the mean. It would be preferred to provide effect sizes as well.

Thank you for the comment. The graphics display has been corrected and demonstrated in standard deviation of the mean.

3. It is not indicated in the figure legends what the red arrows are indicating.

Thank you for the comment. The red arrows indicate the positive cells marked in immunohistochemistry. We will include this information in the figure legends.

4. Please include exact p-values in the manuscript.

Thank you for the comment. We included the exact p values in the graphics.