Author’s response to reviews

Title: CD147 increases mucus secretion induced by cigarette smoke in COPD

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Author’s response to reviews:

Hitendra S Chand, Ph.D. (Reviewer 1): In this study authors report that CD147 and MUC5AC expression were higher in COPD patients with smoking history. Using human bronchial epithelial cells or HBEs in-vitro they show that cigarette smoke exposure increased CD147 levels that induced MUC5AC secretion via MMP9 and p38 MAPK signaling pathways. Therefore, they propose that regulation of CD147 could be a promising target for regulating mucus hyper-secretion in COPD. There are several limitations in this study and the manuscript that preclude the enthusiasm and dilute the importance of these studies, as outlined below:

1. The manuscript language needs an extensive revision to make it as an easy and understandable read.

Answer: We have modified the manuscript.

2. Authors should include lung specimen from COPD subjects without smoking history to substantiate that only smoking is correlated with observed elevated CD147 levels and mucus hyperexpression in the studied population.

Answer: Smoking is the main risk factor of COPD, around 80% COPD are current or former smokers, we plan to focus on the function of CD147 in COPD subjects with smoking. What’s more, in our in vitro experiment, we use cigarette smoke extract to set up mucus hyper-secretion, we want to unify the cause of mucus secretion in vitro and COPD subjects.

It is a good point to consider COPD in non-smokers, since there are less than 20% of COPD are non-smokers. The causes for these non-smoking COPD include alpha-1-antitrypsin deficiency, aging, occupational exposure to dust and chemicals, indoor exposure and outdoor air pollution.

3. In addition, they should also provide an information whether or not these subjects had a reported
history of chronic bronchitis, the phenotype targeted in this study. This phenotype and its relevance in COPD pathogenesis should be included in the introduction and the discussion.

Answer: In the first part of Material and Method, all COPD subjects were the same phenotype of chronic bronchitis and had a history of exposure to cigarette smoking. According to your suggestion, I have provided the relative phenotype of these subjects again in the revised manuscript.

4. The chemical staining used (PAS) for the analyzing airway mucus shows only basic mucopolysaccharides and therefore do not account of acidic mucopolysaccharides. Authors should analyze the Alcian blue stained sections to include the total number of mucous cells in their study.

Answer: As you suggested we stained the specimen with Alcian blue periodic acid Schiff, the results are shown in the revised manuscript.

5. The data presentation of lung tissues analyzed should be reported as mucous cells per mm of basal lamina to avoid inclusion of PAS- and/or MUC5AC-positive submucosal glands. And additional epithelial cell marker should be included to confirm that the signal is only coming from the epithelial cells. For all histological images a magnification or the scale bar should be provided.

Answer: All specimens are taken from sub-pleural parenchyma of the lobe obtained in surgery, at least 5cm far from the diseased regions. Unlike animal lung section it is hard to normalize the site and get the same level of basal lamina in each specimens, we used the percentage of alcian blue positive area in the airway epithelium to represent the mucous cells. We added the bar in all histological images, as showed in the revised manuscript.

6. The in-vitro studies used HBEs and authors should provide their source and origin to help compare the study with other reports.

Answer: HBE cells are immortalized human bronchial epithelial cells and purchased from Fuxiang Biotechnology Co., Ltd. (Shanghai, China).

7. The HBEs are presumed to be grown in a submerged culture model and HBEs do not represent their natural polarity as observed in conducting airways of the lung. Therefore, authors should provide an evidence that CD147, MMP9 and p38 MAPK are involved in mucin regulation of in-vitro differentiated HBEs.

Answer: Air-liquid interface can simulate in vivo growing environment of human bronchial epithelial cell, but air-liquid interface is not suitable in our laboratory. We referred to several journal articles that HBE cells can secrete mucus in medium with 10% fetal bovine serum[1,2], and we used MMP9 and p38MAPK inhibitor separately to detect the function of MMP9 and p38MAPK in mucus regulation.


8. The cigarette smoke referred in the in-vitro experiments should be designated as CS extract and not smoking itself. The concentration of CS extract (10%) used is pretty high compared to other contemporary in-vitro models and should be discussed in the paper.
Answer: We will use cigarette smoke (CS) extract in the revised manuscript. CS extract (100%) was prepared by bubbling smoke from 2 cigarettes in 10 ml of serum-free RPMI-1640 medium at a rate of half a cigarette/min. The pH of the CS extract was adjusted to 7.4, the optical density was determined (0.783±0.03) and CS extract was sterile-filtered through a 0.22 μM filter. The CS extract was always freshly prepared on the day of the experiment. Due to the difference among CS extract preparation protocols, the concentration of CS extract (10%) is only suitable for this preparation procedure.

9. In the experiment reported in Figure 6, please include the non-treated control sample to show the levels of all the proteins analyzed present in an unstimulated HBEs.

Answer: We added the non-treated control sample in Figure 6.

10. Authors fail to discuss their in-vivo findings of elevated CD147, a metalloproteinase inducer levels in their cohort and should compare the data in light of the protease-antiprotease imbalance in the COPD pathogenesis.

Answer: Serum Protein Electrophoresis Test is a regular test in all subjects in our research, the results were normal and there were no alpha-1 antitrypsin deficiency in these subjects.

Sunil K. Nooti, MBBS, Ph.D (Reviewer 2): The study "CD147 improves mucous secretion induced by cigarette smoking in COPD" by Qiao Yu et al., is a correlative study between CD147 expression and MUC5AC in human lung specimens. Apart from the human IHC results, the authors show that CD147 regulates MUC5AC expression through MMP9/p38 MAPK signaling in HBE cells in vitro.

I have two major concerns regarding this study:

1. Even though the correlations in vivo are obvious, it is not clear why smokers with and without COPD have a differential CD147 and MUC5AC expression, even though these two groups have similar overlapping smoking histories (52.8±8.6 vs 60.3±7.4 pack years). Since this is not clear, it is difficult to interpret that their link is causal in nature. When was the diagnosis of COPD made for the third group with respect to age and smoking history pack years? I would strongly recommend the authors to look for more differences between these two groups, which could have resulted in COPD, like are the blood alpha-1-antitrypsin levels different? Were there no other genetic or environmental factors that could have led to COPD in smokers with COPD vs smokers without COPD? Moreover, if all the lung specimens were from resected pulmonary nodules, what were these due to? How are the authors sure that the pathology behind the nodular disease is not contributing to immune cell infiltration or affecting the clearance of mucus even though the regions studied are 5 cm away from the pathology?

Answer: Although there are several genetic or environmental factors that could contribute to the development of COPD, smoking is the main risk factor of COPD, around 80% COPD are current or former smokers. Although there are no significant difference of cigarette history of these two group, there is relatively longer cigarette smoke history in COPD group and another difference maybe the different expression of CD147 during smoke stimulation.

We try to unify genetic and environmental factors in our patients recruited. Patients had no history of exposure to occupational dust and chemicals, indoor and outdoor air pollution. Serum Protein Electrophoresis Test is a regular test in all subjects in our research, the results were normal and there was no alpha-1 antitrypsin deficiency in these subjects mentioned in our manuscript.

We have taken into consideration the representation of these lung specimens; specimens from lung
cancer may have influenced the responses we observed. It is hard to get COPD lung tissue from COPD patients without pulmonary nodules, the only thing we can make sure is that recurrence was rarely seen in 5cm away from the margin of the diseased regions, and we presume that 5cm away from diseased region should be normal.

2. As described by the authors in the background section (citation 17 and references therein), it is already known that there is more CD147 in bronchoalveolar fluid and is expressed predominantly by bronchial epithelium from patients with COPD compared with smokers and nonsmokers. It was also shown here that blocking CD147 led to a decrease in MMP-9 mRNA expression and activity. Since this is already known, what would have been interesting to see was whether there was more MMP9 or phosphorylation of p38MAPK in the lung specimens of smokers with and without COPD. This could have added to the mechanistic basis to some extent. The fact that in vitro experiments in this study use CS, the authors should justify why their results are relevant in light of published literature from in vivo, and why this study is novel.

Answer: There are multifaceted underlying mechanisms in the development of COPD. It seems that the literature has similar results with some part of our research, but the start point of this reference is different from ours. In this reference, they focus on the mechanism of protease-anti-protease imbalance and increased protease activity in COPD. In our work the key is mucus regulation, how CD147 regulate MUC5AC secretion and what molecules are involved in. We think our research is different from this literature

Minor concerns:
1. There are a number of English corrections, which need to be corrected ..... I have only highlighted some here from the first half of page 2. Line number6: "hyper-secretion quantitates" ..... revise
Line 6: "However it is remain unclear".... revise
Line 13 and 26: "In vitro" should be in italics
Line 17: "CD147 involved" .... "is" is missing
Line 28: "silence of" should have been "silencing of"
The manuscript needs professional editing services.

Answer: We have revised our manuscript.

2. Chemical inhibitors used in fig 6 will need to be corroborated with specific inhibition using RNAi for p38 MAPK and MMP9.

Answer: We used siRNA to inhibit CD147 expression, and we wanted to detect the possible CD147-p38MAPK-MMP9 signal pathway. It is an efficient and easy way to use the molecule inhibitors.

3. Scale bars are missing in Fig 1 and 2. If quantitation was done at 200X, the displayed figures should be the same.

Answer: We added scale bars in revised manuscript.

4. The use of the word " improves" in the title is inappropriate…. Should be changed to "increases".

Answer: We changed our title in revised manuscript.