Author’s response to reviews

Title: Standardized Herbal Extract PM014 Alleviates Fine Dust-Induced Lung inflammation in Mice

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Reviewer reports:

Reviewer 1: The study analyzes the effect of a standardized herbal extract, PM104, derived from traditional Korean medicine, in a mouse model of fine-dust induced lung inflammation. The authors show that this extract displays protective anti-inflammatory effects, and this is a potentially interesting finding.

The paper is relatively short at three figures, and would have benefited from a more thorough assessment of inflammation in the mouse model, for example through confirmation of the RT-PCR results with western blots looking at specific inflammation markers.

- Thank you for your interest in our research. It seems that you were referring to an additional experiment on the inflammation markers at the protein level; therefore, we conducted an experiment using ELISA. The markers we identified were IL-1b, IL-6, and IL-17, since among the nine markers tested by RT-PCR in figure 2, these three cytokines appeared to be most affected by PM014. The result showed that the amount of IL-1b, IL-6, and IL-17 was significantly suppressed by PM014 treatment, agreeing with the RT-PCR results (figure 2).

Therefore, we revised the manuscript and added the following paragraphs in result section as; “Effect of PM014 on inflammation-related cytokine production in the lung. ELISA was used to measure inflammation-related cytokine production of lungs. In Figure 4 (A-C), the amount of IL-1b, IL-6, and IL-17 was significantly higher in the PM-treated mice than that in the control group. DEXA group showed a tendency to decrease in comparison to the PM-treated group. However, treatment with 200 mg/kg PM014 reduced significantly amount of these cytokines”.

The mechanism by which the extract exerts is protective effects is also not really addressed by the experiments.
- We appreciate the reviewer’s comment. The mechanism of the protective effects exerted by PM014 was elaborated in previous studies, which in vitro showed that PM014 inhibited TGF-β1-induced epithelial-mesenchymal transition (EMT) and fibroblast activation in alveolar epithelial cells and human lung fibroblasts. Previous studies showed that PM014 targeted TGF-β1 signaling via Smad-dependent pathways and p38 mitogen-activated protein kinases (MAPKs) pathways. Furthermore, PM014 treatment resulted in the downregulation of inflammatory cytokines, chemokines, and fibrosis-related genes and reduction in the transforming growth factor-β1-positive cell population in lung tissue.

Based on the reviewer’s comment, we revised our manuscript as follows:

Although the mechanism of the lung inflammatory response from PM10 is unclear, studies on the impact of PM10 on lung inflammation show decrease in microvascular function and increase in leukocyte, neutrophil, and eosinophil counts. We previously reported the mechanism of the protective effects in the lungs exerted by PM014, which in vitro showed that PM014 inhibited TGF-β1-induced epithelial-mesenchymal transition (EMT) and fibroblast activation in alveolar epithelial cells and human lung fibroblasts. This study showed that TGF-β1 signaling via Smad-dependent pathways and p38 mitogen-activated protein kinases (MAPKs) pathways were the main target of PM014. Furthermore, PM014 treatment resulted in the downregulation of inflammatory cytokines, chemokines, and fibrosis-related genes and reduction in the transforming growth factor-β1-positive cell population in lung tissue.

minor comments: some typos. The Results section is misspelled Result.

- We appreciate the reviewer’s comment and corrected the manuscript.

Reviewer 2: In their work, "Standardized Herbal Extract PM014 Alleviates Fine Dust-Induced Lung inflammation in Mice", Lee et al have evaluated the effect of PM10 on fine dust-induced lung inflammation. They showed that PM014 reduced the damage to the tissues and the infiltration of inflammatory cells, in addition to decreasing the expression of some pro-inflammatory cytokines. Although the results are significant and clearly represented, I'm not that they are enough to completely assume that PM014 indeed alleviates inflammation.

I have several concerns.

1. In figure 1, the authors are showing that PM014 is decreasing the immune cells infiltration. It isn't really clear in the figures. It would be better if the authors refer to the infiltrated immune cells by arrows to clarify the difference between all the conditions, which is not really prominent in the current figures.

- We appreciate the reviewer’s comment and apologize for the lack of clarity in the figures. The figures were modified to be more clearly visible, and the infiltrated immune cells were marked with arrows.

2. In figure 2, the authors have showed the count of the different types of immune cells in the bronchoalveolar fluid. In the Materials and methods, they indicated that the immune cells were stained and counted under the microscopy. How did the authors really distinguish between all the types of immune cells and sort them? Is it only by eye under the microscopy (which is not enough for the assumption) or are there specific stained markers that shall be mentioned? A true vivid proof of immune cells infiltration to the lungs would be through measuring the expression levels of markers for the different immune cells by PCR in the tissue or the use of flow cytometry to detect the different populations in the fluid.
- We appreciate the reviewer’s comment. The experiment was conducted according to the method performed in the previous study on lung inflammation model 5,6. Like the picture below, each immune cell was distinguished. A total of 500 cells per slide were counted and the ratio was plotted. The previous studies which employed the same method on the effectiveness of PM014 are referenced in this paper.

3. The description of the used model is not really clear. The authors said that on days 0,2,7,9 the mice were treated with PM014 followed by PM10. Day 0 corresponds to what? Is it the day of induction of inflammation by PM10 in all groups? And then starting day 2 the (PM10+PM014) groups were re-injected with PM10 following the PM014? A representative scheme of the different groups showing the treatments and timing will be better for clarification.
- We appreciate the reviewer’s comment. As the reviewer suggested, the experimental schedule was added in Figure 1.

4. The authors indicated that they were injecting PM10 following PM014 to study the "prophylactic" effect of PM014. What is the usefulness of this "preventive" investigation clinically? Will people tend to use "preventive" medications for fine dust?
- We appreciate the reviewer’s comment. The reviewer raised an important point regarding the clinical implication of the “preventive” or “prophylactic” effect of PM014. While a long-term effect of fine dust is still under investigation, it seems clear that it will cause various health problems, as much as the fact that it will, to some extent, will be unavoidable to breathe fine dust as a large number of nations are producing fine dust from traffic, construction sites, and manufacturing processes. That said, we may need to turn to the prophylactic effect of medications to minimize the risk of fine dust-induced lung inflammation in the same way as some doctors suggest a low-dose aspirin as prophylactic therapy for cardiovascular risk reduction; consequently, preventive effects of a herbal formula on fine-dust related diseases may take an important role in the future.
Based on the reviewer’s comment, we added the following sentences in the manuscript as follows: Therefore, this study investigated how PM014, a herbal formula that has previously been shown to relieve symptoms related to the lungs such as asthma and COPD7, affects fine dust-induced lung inflammation in a fine dust-induced acute lung injury mouse model. This preventive effect of PM014 on fine dust-related diseases may have an important implication in the future as the impact of fine dust is becoming both substantial and unavoidable.

Minor comments:

5. The resolution of the graphs is really bad, and it's impossible to read all the labels of the X and Y axes.
- We appreciate the reviewer’s comment and apologize again for the low resolution in the figures. The figures were modified to be more clearly visible in the revised manuscript.
6. Figure 3 legend: the authors indicated that "Data are expressed as mean number of cells ± standard error". However, it's a figure for the mRNA level and there are no cells, so this shall be corrected.
- We appreciate the reviewer’s comment and corrected the manuscript as “The ratio of mRNA to β-actin was depicted as mean ± standard error”

7. The authors mentioned that the optimal dosage of PM014 was chosen based on a preliminary dose-response experiment done in a previous publication. It would be useful to mention this idea in the materials and methods section to justify the choice of the dose for any future referring scientist going through the paper.
- We appreciate the reviewer’s comment and corrected the manuscript as below:

Fine dust (PM10)-induced lung inflammation mouse model
Fine dust (100 μg/ea) was used to induce lung inflammation in mice. Female C57BL/6 mice were randomly divided into six groups: control, PM10 group, PM10 + PM014 group, and PM10 + DEXA (dexamethasone, 10 mg/kg). The most efficient concentration of PM014 to elicit inhibitory effects on lung inflammation was investigated in our previous study to be a dose of 200 mg/kg 2. For the evaluation of different doses used in this study those administered therapeutically, we referred to the guidance for industry prepared by the Office of New Drugs in the Center for Drug Evaluation and Research at the Food and Drug Administration 8. The optimal timing for the administration of PM014 was applied based on another preliminary experiment 3, and we decided to use continual treatment of PM014 to examine the significant impact on fine dust-induced inflammation.

8. A small Grammatical Comment "A herb" not "An Herb".
- We appreciate the reviewer’s comment and corrected the manuscript as below:

PM014 is a herbal extract