We would like to thank the editor for efforts on improving the manuscript. All comments and suggestions made by the editor have been addressed in the revised manuscript.

Additionally, we thank the reviewers for the time and effort that they have put into their thoughtful reviews and helpful comments. We have responded to each comment and question below and indicated the modifications in the revised manuscript. Our responses to reviewer comments are bolded below.

Reviewer 1:

Overall, the manuscript is very well written and the protocol is easy to follow. I only have a couple of minor concerns that would help improve clarity.

- R1.C1: Please provide a few words justifying a rationale for the selected 90min time point to assess efferocytosis.
    - ***In vivo*** **models of measuring efferocytosis in the literature using intratracheal (i.t) or oropharyngeal (o.p) instillation of apoptotic cells followed by lavage range from 30 minutes to 2.5 hours post-instillation. In this protocol, and other papers referenced below, bronchoalveolar lavage fluid was collected at the ≥90 minutes post instillation (Lee et al., 2012; Park et al., 2008). This time point was used with the thought of allowing sufficient time for accumulation of signals from the apoptotic cells as well as the migration of alveolar macrophages to the apoptotic cells for engulfment. Additionally, cell differentials as this time point in Park et al., 2008 showed the macrophages did engulf the apoptotic cells and very few thymocytes remained in the airspace. We have now included this explanation in our protocol for clarification to the reader.**

- R1. C2: Day 1, #8, please indicate how long it typically takes for animals to awake from anesthesia and how significant this time is compared to the 90min incubation.
    - **Per the reviewer's suggestion, we indicated in Day 1, #8 description, "Typically, mice should awake following anesthesia in 1-2 minutes" was added to the protocol for clarification. Given how quickly the mice awake, this should not alter the 90 min incubation.**

- R1.C3: Day 2, #8, it will be helpful to indicate the average or range volume of PBS used to lavage an adult mouse lung.
    - **The range of 1X PBS used to lavage an adult mouse lung is between 0.7-1 mL. The lavage protocol we use calculates the lavage volume based on weight of the mouse to ensure that the lungs are not overinflated. As suggested, this has been added to the Day 2 #8 part of the protocol:**

> **"Use a needle to make a slit in the trachea (about ¼ of the way down from the head) and insert a cannula (18 G x 1 ¼ inches) with a syringe pre-loaded with 1x phosphate buffered saline (PBS) (26.25 mL/kg body weight, approximately 0.7-1.0 ml in an 8-10 week old female C57Bl/6J mouse) caudally into the trachea."**

- R1. C4: Day 2, #15 the second sentence is in past tense, check for consistency throughout the protocol.
    - **As suggested, the tense of the protocol was changed from past to present, including Day 2, #15 description:**

      "Each sample (120 µL) is centrifuged onto slides at 12 x g for 3 min, medium acceleration, using a cytocentrifuge. Slides are set aside to dry overnight."

Reviewer 2:

This is an interesting protocol that will likely be of interest to investigators examining macrophages and the resolution of injury.

- R2.C1: Manuscript should be carefully reviewed for language and style. Additional experimental information is needed as described below.
    - **Manuscript was reevaluated for language and writing style to address any inconsistencies.**

- R2. C2: Does this work with cells other than Jurkat? Where are they from?

  Preliminary studies before using Jurkat T cells, our lab isolated thymocytes from 3-4 week old mice to irradiate for instillation. Using apoptotic thymocytes for macrophage efferocytic assays was also successful in other publications (Tao et al., 2015; Bae et al., 2013; Friggeri et al., 2010). Jurkat T cells are used in this protocol because they are a commercially available and inexpensive cell line. Additionally, Jurkat T cells can be kept sterile which minimizes any off-target effects in the airspace. The Jurkat T cells were obtained ATCC and the catalog number is CRL-2899.

- R2.C3: Why were only female mice used?
    - **Only female mice were used in this protocol because it has previously been reported that females display a greater inflammatory response and defects in alveolar macrophage phagocytosis of bacteria after O_3 exposure when compared to males (Fuentes et al., 2019; Cabello et al., 2015; Mikerov et al., 2008). However, future studies in our laboratory include analyzing sex differences in alveolar macrophage efferocytosis.**

- R2.C4: Does the response vary with mouse strain?
    - **We have currently only evaluated C57Bl/6J mice. However, previous reports have indicated that different strains of mice have different response to O_3 exposure, including pulmonary inflammation (Wesselkamper et al., 2001, Kleeberger et al., 2000). Therefore, there could be differences in the amount efferocytosis based on strain exposed. Although these strain screens are very important in understanding susceptibility to environmental lung diseases, we believe the evaluation of strains outside of C57Bl/6J is outside the scope of this protocol. However, we have added in this caveat to our discussion to help the reader understand that the strain chosen may influence the outcome of this assay.**

- R2.C4: Figure 1- more information needs to be provided on how many cells were counted for determination of differentials; the label 24 h post exposure should be included int he figure legend; be consistent with FA versus Filtered Air.
    - **As suggested, the figure legend was edited to clarify methods to:**

      "Figure 1: O_3 exposure induces pulmonary inflammation and injury. C57BL/6J female mice were exposed to filtered air (FA) or 1 ppm O_3 for 3 h. 24 h post exposure, mice were necropsied to analyze pulmonary inflammation and injury (n=6/group). A) Bronchoalveolar lavage (BAL) cell differentials were calculated and epithelial (epi), eosinophils (eos), lymphocytes (lymph), macrophages (Mϕ), and neutrophils (PMN) were identified with atleast 100 cells counted from each slide. B) A representative image of cellular differentials. C) Total protein in the BAL fluid. Data are expressed as ±SEM **p<.01."

- R2.C5: Figure 2- how representative were these data? was it reproducible?
    - **The data used in figure 2 is representative of our studies. Since optimizing the correct energy level and incubation time for the Jurkat T cells, the results show in figure 2 have been reproduced in approximately 6-8 independent studies.**

- R2.C5: Figure 3- how many cells were counted to determine index?
    - **A total of 200 cells were counted to calculate the efferocytic index in this protocol. We have now ensured that this is included in the manuscript.**

- R2. C6: Figure 4- how representative were these data? was it reproducible?
    - **The data used in figure 4 is representative of past suboptimal studies where we had used the different UV bulbs (350nm and not 254nm) which induced more late apoptosis. We have conducted approximately 3 independent suboptimal studies using other sources of UV light that resulted in increased late apoptotic and necrotic Jurkat T cells.**

Reviewer 3:

The authors describe a simple protocol to measure efferocytosis by alveolar macrophages. The approach is relatively simple and straightforward.

- R3. C1: Somehow references got cutoff at # 21 (but they go up to 34 in the text)
    - **We apologize for this oversight. References past #21 are now included in the manuscript and ordered correctly in reference section.**

- R3. C2: line 316, it states: Plating alveolar macrophages can induce both physiological and genetic changes that may alter efferocytosis". I think it may be more appropriate to say "physiological and genomic changes" (or perhaps epigenomic)
    - **We agree with the reviewer and have changed the text on line 316 to say:**

"Plating alveolar macrophages can induce both physiological and genomic changes that may alter efferocytosis."

References:

1 Park, Y. J. *et al.* PAI-1 inhibits neutrophil efferocytosis. *Proceedings of the National Academy of Sciences of the United States of America.* **105** (33), 11784-11789, (2008).

2 Nishi, C., Toda, S., Segawa, K. & Nagata, S. Tim4- and MerTK-mediated engulfment of apoptotic cells by mouse resident peritoneal macrophages. *Mol Cell Biol.* **34** (8), 1512-1520, (2014).

3 Tao, H. *et al.* Macrophage SR-BI mediates efferocytosis via Src/PI3K/Rac1 signaling and reduces atherosclerotic lesion necrosis. *J Lipid Res.* **56** (8), 1449-1460, (2015).

4 Friggeri, A. *et al.* HMGB1 inhibits macrophage activity in efferocytosis through binding to the alphavbeta3-integrin. *Am J Physiol Cell Physiol.* **299** (6), C1267-1276, (2010).

5 Lee, Y. J. *et al.* Apoptotic cell instillation after bleomycin attenuates lung injury through hepatocyte growth factor induction. *Eur Respir J.* **40** (2), 424-435, (2012).

6 Das, A. *et al.* Correction of MFG-E8 Resolves Inflammation and Promotes Cutaneous Wound Healing in Diabetes. *J Immunol.* **196** (12), 5089-5100, (2016).

7 Bae, H. B. *et al.* Vitronectin inhibits efferocytosis through interactions with apoptotic cells as well as with macrophages. *J Immunol.* **190** (5), 2273-2281, (2013).

8 Cabello, N. *et al.* Sex differences in the expression of lung inflammatory mediators in response to ozone. *Am J Physiol Lung Cell Mol Physiol.* **309** (10), L1150-1163, (2015).

9 Mikerov, A. N. *et al.* Sex differences in the impact of ozone on survival and alveolar macrophage function of mice after Klebsiella pneumoniae infection. *Respir Res.* **9** 24, (2008).

10 Fuentes, N., Cabello, N., Nicoleau, M., Chroneos, Z. C. & Silveyra, P. Modulation of the lung inflammatory response to ozone by the estrous cycle. *Physiol Rep.* **7** (5), e14026, (2019).

11 Kleeberger, S. R., Reddy, S., Zhang, L. Y. & Jedlicka, A. E. Genetic susceptibility to ozone-induced lung hyperpermeability: role of toll-like receptor 4. *Am J Respir Cell Mol Biol.* **22** (5), 620-627, (2000).

12 Wesselkamper, S. C., Chen, L. C., Kleeberger, S. R. & Gordon, T. Genetic variability in the development of pulmonary tolerance to inhaled pollutants in inbred mice. *Am J Physiol Lung Cell Mol Physiol.* **281** (5), L1200-1209, (2001).