Re:TAR-20-165

Point-by-point response
We would like to again thank the reviewers for taking the time to review our manuscript and for providing constructive comments and suggestions. Please see our point-by-point responses below in **BOLD**. Changes made to the revised manuscript are highlighted in yellow.

Reviewer: 1

Comments to the Author
This is an interesting review article about the role of the receptor for advanced glycation endproducts (RAGE) in the pathogenesis of Idiopathic pulmonary fibrosis (IPF). The authors have made extensive review of their own work and other previous papers and summarized clearly. This review will help the readers to understand various associations between RAGE and pulmonary fibrosis.

We would like to thank the reviewer for their kind words regarding our review.

Reviewer: 2

Comments to the Author
Here in this review, Dr Perkins and Dr Oury are presenting a concise review of the paradoxical function of RAGE in the physiopathology of IPF. It should be pointed out that the authors have transmitted clearly and briefly the ideas and facts that has been mentioned regarding this molecule. Nevertheless, I have mayor concerns for this manuscript. Briefly, I summarize some of them:

We would like to thank the reviewer for their kind words and appreciate the constructive critiques below.

C1: First of all, I consider that the title could be so much generalist. It is true that one of the objectives of this review is to bring to clues about the controversial role of RAGE in IPF, if it is part of its pathogenesis or a simple “side effect” of the fibrotic progression. However, it is clear that IL-13 has prominent mentioning (almost 4 pages only for explaining its mechanism and the importance of ILC2). It could be so much interesting to include this cytokine or type 2 immunity as a headline.

R1: Thank you for the suggestion. While we agree it could be interesting to include type 2 immunity in the title, we have difficulty in rewording the title to fit this in. If the reviewer may have suggestions, we would be open to considering them for a final version if the revised manuscript is to be published.

C2: Page 3, Line 66: I am not sure that nintedanib and pirfenidone should be spelled with a capital letter at the beginning, because in the case of pirfenidone, Esbriet® should be the commercial name, which has to be named with an initial capital. Could you please check this?

R2: Thank you for pointing this out. Neither nintedanib nor pirfenidone should be capitalized, this has now been corrected.

C3: Page 5, Line 127: There is mention different lung cell lines, specifically lung fibroblasts. Checking the literature that you mentioned, in the references I’ve observed the article of Wang

et al. (J Allergy Clin Immunol. 2015 May;135(5):1154-62.e1-5), which refers to fibrocytes instead of lung fibroblasts. Could you check this reference?

R3: Thank you for pointing out this potential confusion. In the study by Wang et al., fibrocytes were studied, while the study by Xu et al, studied fibroblasts. The sentence has been changed to read as "fibroblasts or fibrocytes" to indicate to readers that the two studies describe two different cell types.

C4: Page 5, Line 128: As commented, these two studies (Inghilleri et al. Pulm Med. 2011;2011:421409; and Morbini et al. Mod Pathol. 2006 Nov;19(11):1437-45) suggested that RAGE and its ligand is increased in the fibroblast foci in the lungs with UIP. However, it is also important to mention that these two references showed a great increment of RAGE in the overall lung from IPF patients by immunohistochemistry. This overexpression of RAGE that those articles describe seems to be contrary to the first statement of this paragraph (Page 5, Line 106). Actually, despite Queisser et al. (Am J Respir Cell Mol Biol. 2008 Sep;39(3):337-45) showed that gene expression of RAGE in lung fibroblast is present, they admitted that RAGE is decreased in alveolar epithelial cells and lung fibroblast from IPF patients compared with lung donors. Also Machahua et al (Respir Res. 2016; 17: 144.) showed that RAGE expression was almost missing in fibroblast foci. How could you explain this disagreement?

R4: Thank you for pointing out these inconsistencies.

Firstly, it seems the main issue with the studies by Inghilleri et al. 2011 and Morbini et al. 2006, is that neither study made a direct comparison of RAGE expression levels in IUP versus healthy lung tissue. While Inghilleri et al. describes control tissue in the methods, I do not see presentation of RAGE expression in these tissues. We agree that the level of RAGE expression seems very high for UIP, especially when compared to the findings of Queisser et al. 2008, which shows strong downregulation. However, there is no normal lung presented in the study by Inghilleri et al.

Similarly, in the study by Morbini et al. 2006, RAGE expression is demonstrated in fibroblastic foci (Fig. 4 of the manuscript), however, there is no direct comparison to normal lung in this figure. In Figure 3 they do show a comparison of RAGE levels in UIP compared to post-obstructive pneumonia and tuberculosis.

I believe the takeaway from these studies is that it is possible that RAGE is still expressed in different compartments of the lung in UIP, notably, in the fibroblastic foci.

Regarding the study by Queisser et al. 2008, while RAGE mRNA levels were reduced in type 2 cells isolated from IPF, levels of RAGE in fibroblasts were unchanged.

We agree, this could be very confusing for readers. The purpose of this section is to point out the inconsistencies in findings regarding RAGE expression in fibroblasts and fibrotic foci, as well as the lack of knowledge regarding overall cellular expression patterns of RAGE in pulmonary fibrosis. We have now more clearly indicated what each of these studies have demonstrated, making note of the inconsistencies and suggest that further research is needed to have a clear understanding of RAGE expression in PF.

C5: Page 6, Line 146: Here I have found another confusing reference. Kyung et al (Int J Clin Exp Pathol. 2013 Dec 15;7(1):221-8) showed that circulating AGE are increased in IPF patients, whereas RAGE is also increased in lung tissue from IPF patients. Although they suggest a

correlation, that seems to be positive. Also Machahua et al (Respir Res. 2016; 17: 144.) did not mention a correlation in circulating stage-AGE, but it was mentioned in “Serum AGE/RAGEs as a potential biomarker in idiopathic pulmonary fibrosis” (Respir Res. 2018 Nov 8;19(1):215).

R5: We apologize for the confusion with this sentence and associated references. I believe we meant to cite Machahua et al. 2018 here. However, upon reviewing, this sentence does not seem necessary and is simply confusing, as it makes mention of the association of circulating sRAGE and AGEs, which is not mentioned again in the text. Therefore, we have removed this sentence. sRAGE levels in IPF are discussed in the section below.

C6: Page 8, Line 196: In order to add novel insight in re-epithelialization and RAGE I suggest to check the reference “The receptor for advanced glycation end-products enhances lung epithelial wound repair: An in vitro study”, Zhai et al. Exp Cell Res. 2020 Jun 15;391(2):112030.

R6: We thank you for suggesting this recent study. We have now made reference to this on page 8 (see lines: 195-201): “*In addition, Zhai et al. recently demonstrated that stimulation of alveolar epithelial cells with AGEs and HMGB1 promoted wound healing capacity and proliferation in a RAGE-dependent manner. In line with this, previous studies demonstrated that RAGE on AEC1 cells binds to collagen and facilitates cell adhesion/spreading. While there are inconsistencies in the role of RAGE in epithelial cell adherence, it also promotes epithelial cell proliferation, migration and wound healing, suggesting a complicated role in re-epithelialization*.”

C7: Page 9, Line 215: Although this section brings new findings regarding the possible mechanism by RAGE could act, I strongly miss a mention of the intricate relationship of IPF and inflammatory response, as well the immune response. It is true that the inflammatory process could lead to a well-established fibrosis, but the clinical practice and the histopathological findings have demonstrated that the inflammation is not the main character of this disease. How can we manage that RAGE could enhance an inflammatory response, such as in some inflammatory lung diseases, with the implication of some Type 2 immunity, in a disease that do not show clear signs of inflammation? Would we be talking about a subclinical inflammation?

R7: Thank you for pointing out this important aspect. It is important to note the complicated and poorly understood role of inflammation in IPF. We have now made note of this in the beginning of this section (see lines: 219-226):
“The role of inflammation in the pathogenesis of IPF has long been a subject of fierce debate. Theoretically, it has been thought that unknown insults and environmental exposures cause injury causes repeated cycles of inflammation and improper repair and remodeling leading to fibrogenesis.[69] However, lack of robust inflammation in subjects with IPF ineffectiveness of corticosteroid treatment challenges this theory.[70] Conversely, various studies have demonstrated that inflammatory cell influx is present during exacerbation and is associated with worse disease outcomes.[1,71] Moreover, proinflammatory and profibrotic cytokines, such as type 2 cytokines IL-4 and IL-13 are elevated in subjects with IPF, as are their receptors.[72-74]”

While inflammation does not appear to be a sole driver of disease in established IPF, we believe it is likely a driving force (concurrently with genetic predisposition and other risk factors). It remains plausible that immune responses to environmental factors or concurrent autoimmune disease for example, significantly contributes to the

development of fibrosis in IPF. As a chronic lung disorder, it seems likely that long-term, repetitive cycles of sub-clinical inflammation and aberrant repair could lead to development of IPF. We suggest that RAGE may contribute to ILC2 responses, which then in turn promote a profibrotic microenvironment, by signaling to profibrotic M2 macrophages and fibroblasts via molecules such as IL-13. It is important to note that in the lungs, ILC2s are very low in abundance, however, they produce copious amounts of type 2 cytokines when activated (by environmental triggers for instance). This suggests the number of cells needed to induce fibrotic responses may not be very easily distinguished histologically.

C8: Page 12, Line 304: Here is still considering the overexpression of RAGE in fibroblast foci with some evidences that could be in the other side, as I mentioned before.

R8: Thank you for pointing this out. We have now changed that sentence to read as follows (lines: 314-316): "*Studies have suggested that RAGE may be expressed in fibroblastic foci in the lungs of subjects with ILD, however more studies are needed to confirm this finding and determine if there is also active signaling*."

C9: Page 16, Line 406: In this sentence, I do not observe the contrasted wording. Loss of RAGE could be a secondary mechanism derived from alveolar loss and its gene expression could also decrease by aberrant molecular mechanism. In IPF, the reorganization of the alveolar parenchyma of the fibrotic process is progressive and heterogeneous where the remaining hypertrophy/reactive alveolar epithelial cells had an important role in the maintenance of the pro-fibrotic environment; it is here where a decrease of RAGE could be determinant of an aberrant re-epithelialization. Thus, these two mechanisms that seem to cancel each other, might be supplementary one to another. In the second place, the loss of membrane RAGE could be perfectly related to the lessening in sRAGE, considering that the main source of the soluble isoform is the cleavage of the membrane isoform (Hudson et al. FASEB J. 2008 May;22(5):1572-80), and less than the 7% of sRAGE is endogenous secreted.

R9: Thank you for making this point. We agree that two divergent mechanisms could be at work leading to decreased RAGE levels in IPF. We have made adjustments to this sentence, which hopefully now makes the point that there decreased RAGE levels could induced as a secondary to injury or through direct down-regulation. The sentences now reads (lines: 418-424) "This suggests that loss of RAGE may be in effect a *casualty* of alveolar injury and loss of normal lung architecture in IPF. On the other hand, developmental studies indicate that RAGE contributes to alveolarization in the lungs, suggesting alternative mechanisms or genetic errors could *causally* lead to reduced RAGE expression and in turn, aberrant re-epithelialization".

C10: Page 17, Line 412: Missing reference.

R10: References have been added.

C11: Page 17, Line 413: The problem with associating the RAGE-ligand interaction with the progressive fibrogenesis is the amount of reports that suggested that RAGE is decreased in IPF; thus in a therapeutic approach, when could RAGE inhibitor or antagonist be administrated? It is true that is so much tentative suggesting a role in the pathogenesis of the disease, as another driver process that may trigger the fibrotic response, and that is a good point. The only question about that is if RAGE has a role in progressive fibrosis, why there is no reported changes of RAGE in acute exacerbations for IPF?

R11: We agree that it is difficult to indicate that RAGE-signaling could be a driving factor in the progressive phases of IPF, given the amount of data that shows RAGE is decreased (overall) in the lungs of subjects with IPF. However, there are simply not enough studies that investigated RAGE levels and/or signaling during exacerbations and progressive phases of disease. Moreover, it is also difficult to determine if RAGE signaling could still be active and be a force that promotes fibrogenesis in response to injury during exacerbations and in fibroblastic foci. Because RAGE is so abundantly expressed in the type 1 alveolar epithelium, it is difficult to determine relative expression levels using general immunofluorescence or IHC approaches. There is a great need for more studies to determine the overall changes in RAGE expression throughout the different compartments and cell-types of the lungs in IPF. As for a therapeutic approach, RAGE inhibition could be helpful in more accelerated disease to slow the progression of disease, but not to reverse it. However, too much remains unknown at this time. Some key questions that need to be answered are: is RAGE decreased in IPF due to loss of alveolar epithelium or aberrant down-regulation to hinder inflammatory responses? Is RAGE involved in re-epithelializtion and if so, does down-regulation to slow inflammation inhibit proper re-epithelialization? Lastly, although alveolar epithelial expression is decreased in IPF, is RAGE-signaling still active at sites of injury and active fibrogenesis.

Reviewer: 3

Comments to the Author
C1: A very well written and comprehensive review on the role of RAGE in the pathophysiology of IPF. The concept of progressive fibrotic ILD such as rheumatoid arthritis and systemic sclerosis, is starting to be recognised where their clinical behaviour are very similar to IPF. Are there any data available on RAGE levels on such ILD cases and are they any different from IPF?

R1: Firstly, we thank you for your kind words on this review. To our knowledge, whcle RAGE and its ligands (e.g. S100A8/A9) have been associated with systemic sclerosis and rheumatoid arthritis, there does not be any reports linking them to associated ILD. Future studies on this matter would likely be beneficial to current understanding.