Author’s response to reviews

Title: The overexpression of peroxiredoxin-4 affects the progression of idiopathic pulmonary fibrosis

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Author’s response to reviews:

BMC Pulmonary Medicine

Editor-in-Chief

David Noel O'Dwyer
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Prof. Dr. O'Dwyer,

Thank you very much for giving us the opportunity to respond to the comments of the reviewers regarding our manuscript, “Peroxiredoxin-4: a possible diagnostic biomarker for early diagnosis of acute exacerbation of idiopathic pulmonary fibrosis” by Hanaka et al.

We have revised our manuscript according to the comments below, and we have uploaded the response letter file including a table and figures as a supplementary material, so please check it.

Respectfully yours,

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RESPONSE TO REVIEWER 1

Major:

1. The authors examined serum biomarkers from IPF and AE-IPF patients and found elevated PRDX4 (Figure 1). This raises the question as to whether there are differences between PRDX4 in IPF and normal donor controls. A recent publication (Elko et al) suggests that there is differences in mRNA expression of PRDX4 between IPF and normal donor controls, but they do not measure protein expression. Addition of this important control in necessary to understand the implications of PRDX4 as a biomarker of pulmonary fibrosis.
Response: We appreciate for your comments and completely agree with your opinion. We have now added the data of the 15 healthy volunteers with some descriptions to the Background and Discussion section as follows:

Before
Page 6, line 92:
Methods: Serum PRDX4, KL-6, SP-D, and LDH levels in 51 and 38 patients with stable IPF (S-IPF) and AE-IPF were retrospectively assessed using enzyme-linked immunosorbent assay.

After
Page 6, line 99:
Methods: Serum levels of PRDX4 protein, KL-6, SP-D and LDH in 51 patients with stable IPF (S-IPF), 38 patients with AE-IPF and 15 healthy volunteers were retrospectively assessed using enzyme-linked immunosorbent assay.

Before
Page 6, line 98:
Serum PRDX4, KL-6, SP-D, and LDH levels in patients with AE-IPF were significantly higher than those in patients with S-IPF.

After
Page 6, line 106:
Serum levels of PRDX4 protein, KL-6, SP-D and LDH in patients with S-IPF and AE-IPF were significantly higher than those in healthy volunteers, and those in AE-IPF patients were the highest among the three groups.
however, the role of PRDX4 in the pathogenesis and progression of IPF is still unclear.

In addition, an increase in the PRDX4 mRNA expression in the lung tissue of patients with interstitial lung disease was reported [18], but the role of PRDX4 in the pathogenesis and progression of IPF is still unclear.

In the present study, we compared the serum PRDX4 protein level in patients with stable IPF (S-IPF), AE-IPF and healthy volunteers to evaluate the significance of PRDX4 in patients with IPF.

…between April 2010 and December 2016 were analyzed for serum PRDX4 levels.
After
Page 10, line 168:

…between April 2010 and December 2016 were analyzed for serum PRDX4 protein level. In addition, serum samples of 15 healthy adult volunteers (32-47 years old) were also collected.

Before
Page 15, line 256:

Continuous variables were compared using the Mann–Whitney U test and categorical variables using the Chi-square or Fisher exact test, as appropriate.

After
Page 16, line 279:

Continuous variables were compared using the Mann–Whitney U test or Kruskal-Wallis test, and categorical variables were compared using the chi-square test or Fisher’s exact test, as appropriate.

Before
Page 15, line 269:

We analyzed the blood samples of 51 and 38 patients with S-IPF and AE-IPF, respectively.

After
Page 17, line 292:

We analyzed the blood samples of 51 patients with S-IPF, 38 patients with AE-IPF and 15 healthy volunteers.
Serum PRDX4, KL-6, SP-D, and LDH levels in patients with AE-IPF were all significantly higher than those in patients with S-IPF, respectively (Figure 1).

The serum levels of PRDX4 protein, KL-6, SP-D and LDH in patients with S-IPF were all significantly higher than those in healthy volunteers (Figure 1), and those in AE-IPF patients were all significantly higher than those in S-IPF patients.

Fig. 1 Serum PRDX4, KL-6, SP-D, and LDH levels in patients with S-IPF and AE-IPF. Serum PRDX4 protein, KL-6, SP-D, and LDH levels in patients with AE-IPF were significantly higher than those in patients with S-IPF.

Figure 1. Serum PRDX4 protein, KL-6, SP-D and LDH levels in healthy volunteers and patients with S-IPF and AE-IPF. Serum PRDX4 protein, KL-6, SP-D and LDH levels were significantly higher in S-IPF patients than those in healthy volunteers. In addition, these levels in AE-IPF patients were significantly higher than those in patients with S-IPF.

(Please see the response letter file)
2. Since PRDX4 is both intracellular and secreted, what is the correlation between the amount secreted and the amount retained in the cells? The authors demonstrate elevated PRDX4 in the serum of AE-IPF patients (Figure 1) and in the serum and BALF of bleomycin challenged mice (Figure 5). However, the immunofluorescence staining of the lung epithelial cells and macrophages in Figure 5B does not seem to be different between the saline and bleomycin challenged Tg mice. Understanding the relative amount of intracellular and secreted PRDX4 in specific lung cell types is important in understanding the role of PRDX4 in mediating pulmonary fibrosis. In addition, isolated cell (rather than lung sections) or use of light field images would be helpful in distinguishing the location of PRDX4 via IF.

Response: In our human study, we demonstrated that the serum PRDX4 protein level was higher in AE-IPF patients than in S-IPF patients. However, as you suggested, it is important to clarify the correlation between the secretion and preservation ratios of PRDX4 in each cell in addition to identifying the types of cells specifically secreting PRDX4 in IPF patients. Although PRDX4 has been reported to be secreted in response to stimulation, such as inflammation and oxidative stress, in vivo (Schulte J, et al. BMC Med. 2011; 9: 137.), the specific cell types that secrete PRDX4 and the ratio of intracellular and secreted PRDX4 have been unclear.

As shown in Figure 5b, high-power field images of the lung showed that PRDX4 is expressed by alveolar macrophages and epithelial cells in Tg-saline and by alveolar macrophages, epithelial cells and myofibroblasts in Tg-BLM. However, the differences in the conditions (e.g. time from staining to shooting, exposure, etc.) made the quantitative comparison of the amount of PRDX4 in each cell between Tg-saline and Tg-BLM by immunofluorescent staining difficult. Unfortunately, we were unable to perform isolated cell evaluations and light field imaging to clarify these issues in the present study, although we hope to resolve these issues in the next step.

We have now mentioned this as a limitation as follows:
However, PRDX4 expressions in the normal and inflamed human lungs are still unclear. Immunohistochemistry of murine lungs demonstrated PRDX4 expression in alveolar macrophages and alveolar epithelial cells in Tg-BLM. This location is similar to that of PRDX1 and PRDX6 expressions observed in the fibrotic murine lungs [18].

However, the PRDX4 expression in the normal and inflamed human lungs is still unclear, and the types of cells that secrete PRDX4 as well as the ratio of secretion and intracellular PRDX4 in each cell type have been unclear in patients with IPF. In the present study, immunohistochemistry of murine lungs demonstrated the PRDX4 expression in alveolar macrophages and alveolar epithelial cells in Tg-BLM, although the amount and ratio of secreted and intracellular PRDX4 in each cell type remained unclear. This location is similar to that of the PRDX1 expressed in fibrotic murine lungs [16].

3. The authors state in line 163 that they measured PRDX4 levels in serum and BALF from humans and mice, but Figures 1 and 2 are both serum measurements (Line 626 and 630). How do levels of PRDX4 differ between BALF and serum of IPF and AE-IPF patients?

Response: We apologize for our misleading descriptions. We did not measure the PRDX4 protein levels in human BALF but those in murine BALF. Therefore, we measured the PRDX4 protein in BALF of S-IPF patients (n = 14) and AE-IPF patients (n = 10) who underwent BAL at the diagnosis of their disease. As a result, we noted no significant differences in the levels between patients with S-IPF and AE-IPF, nor were any significant correlations noted between the serum and BALF PRDX4 protein levels (Spearman's rank correlation coefficient, r = 0.218; p = 0.296).

These data have now been added as additional files, and the manuscript was revised as follows:
Before

Page 16, line 277:

Serum PRDX4, KL-6, SP-D, and LDH levels in patients with AE-IPF were all significantly higher than those in patients with S-IPF, respectively (Figure 1).

After

Page 17, line 306:

The serum levels of PRDX4 protein, KL-6, SP-D and LDH in patients with S-IPF were all significantly higher than those in healthy volunteers (Figure 1), and those in AE-IPF patients were all significantly higher than those in S-IPF patients. In contrast, the BALF PRDX4 protein level did not differ significantly between the patients with S-IPF and AE-IPF (Additional Figure S1), nor were any significant correlations noted between the serum and BALF PRDX4 protein levels in either case (Additional Figure S2).

Before

Page 25, line 436:

This study has several limitations. First, the human study was a single-center retrospective study with a limited number of patients with S-IPF and AE-IPF for detecting serum PRDX4 levels.

After

Page 27, line 478:

This study has several limitations. First, the human study was a single-center retrospective study with a limited number of patients with S-IPF and AE-IPF for detecting serum and BALF PRDX4 protein levels.

Before

(None)
Figure S1. BALF PRDX4 protein levels in patients with S-IPF and AE-IPF. The BALF PRDX4 protein levels did not differ significantly between patients with S-IPF and AE-IPF.

Figure S2. The relationship between the serum and BALF PRDX4 protein levels in patients with IPF. There were no significant correlations between the serum and BALF PRDX4 protein levels (Spearman's rank correlation coefficient, r = 0.218, p = 0.296).

In addition, the authors state that some of the S-IPF patients also had an acute exacerbation. Additional information regarding the timing of sample collection and the sequence of samples are needed to understand Figure 2. In every case, was the S-IPF sample collected before the AE-IPF sample?

Response: We apologize for our incomplete descriptions. Serum samples were collected at the time of the diagnosis of S-IPF and at the time of AE in patients with AE-IPF, but among the 51 patients with S-IPF, 9 developed AE during the observation, so the serum samples of these 9 patients were collected at both the time of the diagnosis of S-IPF before the development of AE as well as at the time of AE development. We have now clarified this point as follows:
For patients with S-IPF in whom AE-IPF occurred during the follow-up period, the serum samples were obtained from both stable and AE states.

For patients with S-IPF in whom AE-IPF occurred during the follow-up period, the serum samples were obtained both in the stable state of IPF (as S-IPF) prior to AE-IPF and at the time of AE of IPF (as AE-IPF).

We analyzed the blood samples of 51 and 38 patients with S-IPF and AE-IPF, respectively.

We analyzed the blood samples of 51 patients with S-IPF, 38 patients with AE-IPF and 15 healthy volunteers. Among them, BALF samples were also evaluated in 14 S-IPF and 10 AE-IPF patients. During the observation period, 9 of the 51 S-IPF patients developed AE, and the serum samples of these 9 patients were collected in both the stable state before AE developed (as S-IPF) and also at time of AE development (as AE-IPF).

4. In Figure 5c, d, e, the authors state that they are using WT and Tg, but the results say Tg-BLM and WT-BLM. Does the ELISA pick up human and mouse PRDX4? Otherwise, since this is a human Tg overexpressing mouse, shouldn't the control be a Saline-Tg rather than a WT-BLM which doesn't have the human transgene overexpressed? Is the system leaky, and how much PRDX4 is expressed at baseline?
Response: We apologize if our depictions were difficult to follow. The human PRDX4 ELISA kit we used actually detects not only human PRDX4 but also mouse PRDX4, because of their highly homologous amino-acid sequences. The baseline mRNA expression for human PRDX4 in wild-type and PRDX4 transgenic mice has been newly added as Figure 5. We also mentioned this in the limitation and modified the text in the Methods, Results, and Figure Legends as show below. In addition, we apologize for our errors in the p-value descriptions and have now corrected these values in Figures 5c, d, and e.

Before

Page 10, line 177:

This solution or 40-μL sterile saline alone (control group) was intratracheally instilled in both WT and PRDX4-transgenic mice. Body weights were recorded at 0, 3, 7, 14, and 21 days after the intratracheal instillation. On day 21, the mice were sacrificed under deep anesthesia with sodium pentobarbital (50 mg/kg, i.p.).

The number of treated mice was as follows: saline-treated WT mice (WT-saline) (n = 5), saline-treated Tg mice (Tg-saline) (n = 5), BLM-treated WT mice (WT-BLM) (n = 14), BLM-treated Tg mice (Tg-BLM) (n = 14). The number of mice that survived until day 21 and were evaluated was as follows: WT-saline (n = 5), Tg-saline (n = 5), WT-BLM (n = 12), Tg-BLM (n = 7). In order to avoid the influence on other experimental results by the BALF operation, mice were prepared for performing the BALF operation. The number of treated mice for BALF operation was as follows: WT-saline (n = 5), Tg-saline (n = 5), WT-BLM (n = 14), Tg-BLM (n = 14). The number of mice that survived until day 21 and underwent BALF operation was as follows: WT-saline (n = 5), Tg-saline (n = 5), WT-BLM (n = 12), Tg-BLM (n = 6).

After

Page 11, line 195:

This solution or 40-μL sterile saline alone (saline group) was intratracheally instilled in both WT and Tg mice. Body weights were recorded at 0, 3, 7, 14 and 21 days after the intratracheal instillation. On day 21, the mice were sacrificed under deep anesthesia with sodium pentobarbital (50 mg/kg, i.p.). Separately, a group sacrificed under deep anesthesia on day 0 without any intratracheal instillation was provided in order to evaluate the human PRDX4 mRNA and protein levels at baseline (baseline group).
The numbers of mice were as follows: WT mice at baseline (WT-baseline) (n = 5), Tg mice at baseline (Tg-baseline) (n = 5), saline-treated WT mice (WT-saline) (n = 5), saline-treated Tg mice (Tg-saline) (n = 5), BLM-treated WT mice (WT-BLM) (n = 14) and BLM-treated Tg mice (Tg-BLM) (n = 14). The number of mice that survived until day 21 among the saline- and BLM-treated mice was as follows: WT-saline (n = 5), Tg-saline (n = 5), WT-BLM (n = 12) and Tg-BLM (n = 7). Furthermore, to avoid the influence of BAL on other experimental results, the following numbers of mice were prepared for the BALF analysis: WT-baseline (n = 5), Tg-baseline (n = 5), WT-saline (n = 5), Tg-saline (n = 5), WT-BLM (n = 14) and Tg-BLM (n = 14). The number of mice that survived until day 21 among the saline- and BLM-treated mice for the BALF analysis was as follows: WT-saline (n = 5), Tg-saline (n = 5), WT-BLM (n = 12) and Tg-BLM (n = 6).

Before

Page 17, line 313:

In addition, both serum and BALF PRDX4 protein levels (Figure 5c and d) and PRDX4 mRNA levels in the lung (Figure 5e) of Tg-BLM were significantly higher than those in the lungs of WT-BLM.

After

Page 20, line 347:

In addition, the BALF human PRDX4 protein level in Tg-BLM was significantly higher than in WT-BLM (Figure 5c). The serum human PRDX4 protein level in Tg-baseline, Tg-saline and Tg-BLM was significantly higher than in the WT mice (Figure 5d). Furthermore, the human PRDX4 mRNA levels in lungs of Tg-baseline, Tg-saline and Tg-BLM were significantly higher than in the WT mice (Figure 5e).

Before

Page 25, line 438:

Second, a cross-reaction of the anti-human PRDX4 antibody with mouse PRDX4 can be observed [24]; therefore, immunohistochemical staining of lungs of WT mice revealed human PRDX4-positive cells because amino-acid sequences of human and mouse PRDX4 are highly homologous, resulting in the cross-reaction of the anti-human PRDX4 antibody with mouse PRDX4 [27].
Second, cross-reaction of the anti-human PRDX4 antibody with mouse PRDX4 can be observed, as the amino-acid sequences of human and mouse PRDX4 are highly homologous [25]; therefore, immunohistochemical staining of lungs of WT mice revealed human PRDX4-positive cells.

Fig. 5 PRDX4 expression in mice. …c and d) ELISA for assessing serum and BALF human PRDX4. BALF and serum human PRDX4 levels were markedly higher in Tg-BLM than those in WT-BLM. e) Human PRDX4 mRNA levels in murine lung homogenates. Human PRDX4 mRNA levels were markedly higher in Tg-BLM than those in WT-BLM.

Figure 5. PRDX4 expression in mice. …c and d) An ELISA for assessing the BALF and serum human PRDX4 protein levels. The BALF and serum human PRDX4 protein levels were markedly higher in Tg-BLM than in WT-BLM. The serum human PRDX4 protein levels in Tg-baseline and Tg-saline mice were significantly higher than in the WT mice. e) The human PRDX4 mRNA levels in murine lung homogenates. The human PRDX4 mRNA levels were markedly higher in Tg-baseline, Tg-saline and Tg-BLM than in WT mice.

(Please see the response letter file)
After

Figure 5c, d, e:

(Please see the response letter file and Figure file)

5. PRDX4 has a regulatory role in the activation of NF-κB; however there is little to no difference in expression of NF-κB regulated cytokines in the WT and Tg mice after saline or bleomycin challenge. One possibility is that the cytokine signal is diluted in the whole lung homogenate. Additional experimentation using the BALF, serum samples, or isolated cells are needed to characterize the role of PRDX4 in regulating cytokine and chemokine production after bleomycin challenge.

Response: In the present study, although there were no significant differences in the expression of NF-κB-regulated cytokines between the two groups, the IL-17A mRNA expression was significantly higher in Tg-BLM than in WT-BLM mice (Figure 7f). IL-17A activates NF-κB (Immunity. 2011;34:149-62), but PRDX4 suppresses the activation of NF-κB (J Biol Chem. 1997;272:30952-61); therefore, our results might be partly explained by the conflicting actions of PRDX4 and IL-17A. As you mentioned, it would be very interesting and important to confirm the expression of NF-κB-related molecules in each cell type in the lung. However, we unfortunately cannot prepare this animal experiment using PRDX4-Tg mice at present because the co-author who created and maintained the PRDX4-Tg mouse model had their job transferred to another facility. Please permit us to address this issue in another study in the future.

We have now modified the Discussion to reflect this point as follows:

Before

Page 23, line 404:

Therefore, PRDX4-induced overexpression of IL-17A may play an important role in the pathogenesis and progression of BLM-induced pulmonary inflammation and fibrosis. Further information regarding the relationship between PRDX4 and IL-17A in the lungs in patients with IPF is necessary to better understand the pathogenesis and progression of IPF.
Therefore, the PRDX4-induced overexpression of IL-17A may play an important role in the pathogenesis and progression of BLM-induced pulmonary inflammation and fibrosis. The expression of NF-κB-regulated cytokines in the WT and Tg mice after saline or BLM challenge was not markedly different in the present study, but both the suppressive effect of PRDX4 on NF-κB [13] and the activating effect of IL-17A on NF-κB [39] might partly explain the conflicting findings seen in the expression of NF-κB-regulated cytokines in the WT and Tg mice after saline or BLM treatment.

Minor:

1. According to the methods, the authors use only male mice for the experiments. Limitation on the interpretation of the data need to be included since only male mice were used for the experiments.

Response: In the present study, we only used male mice, similar to our previous research, so the differences between male and female PRDX4 transgenic mice were not verified. We intend to address this point in the future. For now, we have mentioned this as a limitation as follows:

Before

Page 25, line 440:

... human PRDX4-positive cells because amino-acid sequences of human and mouse PRDX4 are highly homologous, resulting in the cross-reaction of the anti-human PRDX4 antibody with mouse PRDX4 [27].
... human PRDX4-positive cells. Third, only male mice were used in this study, similar to our previous research [19, 20, 24, 25], and we were unable to evaluate the gender differences in the pathogenesis of IPF in Tg mice.

2. Figure 3 is difficult to read because of similarities between the lines. The use of color or other symbols would greatly

Response: As suggested, we have modified Figure 3 as follows:

Before

(Please see the response letter file)

After

(Please see the response letter file and Figure file)

RESPONSE TO REVIEWER 2

(1) In the Abstract and Background/Discussion sections of the manuscript (MS), the authors have summated that serum PDRX4 "is" or "can be" a useful biomarker in distinguishing S-IPF from AE-IPF. This is a premature observation, further validation of the role of PRDX4 is required. This text should be rephrased in the respective sections.

Response: As suggested, we have now changed the title and sentences as follows:
Before

Title:

Peroxiredoxin-4: a possible diagnostic biomarker for early diagnosis of acute exacerbation of idiopathic pulmonary fibrosis

After

Title:

The overexpression of peroxiredoxin-4 affects the progression of idiopathic pulmonary fibrosis

Before

Page 7, line 114:

Conclusions: PRDX4 is associated with the aggravation of inflammatory changes and fibrosis in the pathogenesis of IPF, and serum PRDX4 is a useful marker distinguishing AE-IPF from S-IPF in the clinical practice for patients with IPF.

After

Page 7, line 114:

Conclusions: PRDX4 is associated with the aggravation of inflammatory changes and fibrosis in the pathogenesis of IPF, and serum PRDX4 may be useful in clinical practice of IPF patients.

Deleted sentences

Page 20, line 357:

These results suggest that serum PRDX4 can be a clinically useful marker for judging AE-IPF. Moreover, PRDX4 may have roles in aggravating the pathogenesis of AE-IPF.
The molecular weight of these biomarkers is smaller than that of KL-6 (> 200 kDa) [30], and considering that molecular weight is associated with biomarker profiles [10, 28], small molecular weight of PRDX4 (34 kDa) [17] may explain its better profile as a biomarker for detecting AE-IPF.

The molecular weight of these biomarkers is smaller than that of KL-6 (> 200 kDa) [31], and considering that the molecular weight is associated with biomarker profiles [10, 29], the small molecular weight of PRDX4 (34 kDa) [20] may explain its better profile as a marker for detecting AE-IPF.

The results of the present study suggest that PRDX4 expression is associated with an exacerbation of lung fibrosis, and serum PRDX4 can be a useful indicator for IPF, particularly for AE-IPF. Further studies are warranted to enable better understanding of the role of PRDX4 in fibrotic lung diseases.

The results of the present study suggest that PRDX4 is associated with the aggravation of IPF, and serum PRDX4 may be useful in clinical practice of IPF patients. Further studies are warranted to enable a better understanding of the detailed role of PRDX4 in IPF.
(2) In the Abstract and Background/Discussion sections of the MS, the authors report that other members of the PRDX have been investigated in IPF (namely, PRDX1 and PRDX6). The authors should note that PRDX1 was investigated in a murine bleomycin model of IPF and has not been investigated in IPF patients (Ref 18 in MS). PRDX6 has been investigated previously in a paraquat-induced model of lung injury (PRDX6-KO mice died at 4 days post-treatment; the bleomycin model is routinely a 21-day model). This murine study (see Ref 19 in MS) has no relevance to IPF. The authors should address these anomalies regarding Refs 18 and 19 within the MS. Furthermore, a study in 2008 by Vuorinen et al. (10.1369/jhc.2008.951806) investigated the role of PRDX2 in lung tissue biopsies from IPF patients, which showed that results suggest that Prx II oxidation does not relate to the pathogenesis of IPF/UIP and that Prx II, PDGFRs, and proliferating cells colocalize in the IPF/UIP lung. The MS should be updated to include this reference also.

Response: As suggested, we have now revised our manuscript as follows:

Before

Page 8, line 134:

Human PRDX4 is the only secretory isoform that exists in both intra- and extracellular spaces [13, 14] and is ubiquitously synthesized and is abundantly expressed in various organisms [15].

After

Page 8, line 144:

Human PRDX4 is the only secretory isoform that exists in both intra- and extracellular spaces [13, 14] and is ubiquitously synthesized and abundantly expressed in various organisms [15]. Regarding the role of each PRDX in IPF, a protective role of PRDX1 in bleomycin (BLM)-induced pulmonary fibrosis (PF) mice was reported [16]. In addition, the co-localization of PRDX2 with platelet-derived growth factor receptors (PDGFRs) and proliferating cells in human lung tissue in patients with IPF/usual interstitial pneumonia (UIP) was also reported [17].
Among the members of the PRDX family, PRDX1 is expressed in alveolar macrophages, and PRDX6 is expressed in bronchial and alveolar epithelial cells in the BLM-induced PF murine model [18]. However, PRDX4 expressions in the normal and inflamed human lungs are still unclear. Immunohistochemistry of murine lungs demonstrated PRDX4 expression in alveolar macrophages and alveolar epithelial cells in Tg-BLM. This location is similar to that of PRDX1 and PRDX6 expressions observed in the fibrotic murine lungs [18].

We deleted the underlined part in the above section (please see the response letter file) and added the following sentences:

Among the members of the PRDX family, PRDX1 is expressed in alveolar macrophages in the BLM-induced PF murine model [16]. However, the PRDX4 expression in the normal and inflamed human lungs is still unclear, and the types of cells that secrete PRDX4 as well as the ratio of secretion and intracellular PRDX4 in each cell type have been unclear in patients with IPF. In the present study, immunohistochemistry of murine lungs demonstrated the PRDX4 expression in alveolar macrophages and alveolar epithelial cells in Tg-BLM, although the amount and ratio of secreted and intracellular PRDX4 in each cell type remained unclear. This location is similar to that of the PRDX1 expressed in fibrotic murine lungs [16].

(3) In the Methods/Human Study section (page 9 of MS), the authors state that they collected serum from IPF patients from April 2010 to December 2016. On page 15, the authors state that they analyse blood samples from n=51 S-IPF and n=38 AE-IPF patients (Figure 1). Did the authors just collect serum at diagnosis or do they have follow-up serum?

Response: We apologize for our incomplete descriptions. Serum samples were collected at the time of the diagnosis of S-IPF and at the time of AE in patients with AE-IPF, but among the 51 patients with S-IPF, 9 developed AE during the observation, so the serum samples of these 9 patients were collected at both the time of the diagnosis of S-IPF before the development of AE as well as at the time of AE development. We have now clarified this point as follows
Before
Page 9, line 156:

For patients with S-IPF in whom AE-IPF occurred during the follow-up period, the serum samples were obtained from both stable and AE states.

After
Page 10, line 172:

For patients with S-IPF in whom AE-IPF occurred during the follow-up period, the serum samples were obtained both in the stable state of IPF (as S-IPF) prior to AE-IPF and at the time of AE of IPF (as AE-IPF).

Before
Page 15, line 269:

We analyzed the blood samples of 51 and 38 patients with S-IPF and AE-IPF, respectively.

After
Page 16, line 292:

We analyzed the blood samples of 51 patients with S-IPF, 38 patients with AE-IPF and 15 healthy volunteers. Among them, BALF samples were also evaluated in 14 S-IPF and 10 AE-IPF patients. During the observation period, 9 of the 51 S-IPF patients developed AE, and the serum samples of these 9 patients were collected in both the stable state before AE developed (as S-IPF) and also at time of AE development (as AE-IPF).

(4) On page 16, the MS, the authors state that "during the observation period" 9 patients with S-IPF developed AE-IPF (Figure 2). What was the duration of the observation up period? This should be clarified in the MS.
Response: Thank you very much for your comments. We have now revised our manuscript as follows:

Before

Page 16, line 282:

During the observation period, nine patients with S-IPF subsequently progressed to AE-IPF.

After

Page 18, line 315:

Nine patients with S-IPF subsequently progressed to AE-IPF, and the interval until the diagnosis from S-IPF to AE-IPF ranged from 62 to 1,373 (median: 552) days.

(5) In Table 1, the authors give the baseline characteristics for the S-IPF and AE-IPF patients. The MS would be improved if they also included values for FVC and DLco. If the authors have these PFT values, they should include decline in FVC over the duration of the study in the MS. Also, was there any correlation between decline in FVC and serum levels of PRDX4?

Response: Thank you very much for your valuable suggestions. FVC and DLco were not measured in all patients, and only the data available at the diagnosis of S-IPF and AE-IPF were included in Table 1. However, more than half of the patients in this study did not undergo a second pulmonary function test during the follow-up period, so unfortunately, the evaluation of the relationship between the change in the pulmonary function and serum PRDX4 level was difficult. According to the comments from Reviewer 1, we added the data of the 15 healthy volunteers to Table 1, as follows:
Before

Table 1 Characteristics of patients with S-IPF and AE-IPF

(Please see the response letter file)

After

Table 1. Characteristics of healthy volunteers and patients with S-IPF and AE-IPF

(Please see the response letter file and Manuscript file)

Before

Page 15, line 270:

Compared with the S-IPF group, significantly lower arterial partial pressure of oxygen/fraction of inspired oxygen (PaO2/FiO2) ratio and significantly higher alveolar–arterial difference of oxygen (A-aDO2) were observed in the AE-IPF group, and the overall survival of the AE-IPF group was significantly worse than that of the S-IPF group after diagnosis.

After

Page 17, line 297:

Compared with the S-IPF group, the AE-IPF group showed a significantly lower arterial partial pressure of oxygen/fraction of inspired oxygen (PaO2/FiO2) ratio, significantly higher alveolar–arterial difference of oxygen (A-aDO2), significantly lower FVC and significantly lower diffusing capacity of the lungs for carbon monoxide (DLCO), and the overall survival of the AE-IPF group was significantly worse than that of the S-IPF group after the diagnosis.
(6) In the murine lung homogenates from the bleomycin model, only CCL2 and IL-17A are increased in PRDX4-Tg mice+Bleomycin compared with PRDX4-Tg mice+saline. No other significant differences were found in cytokine/growth/pro-fibrotic factors. The authors quantitated levels of IL-1b, IL-6 and TNF-a transcription, as per serum analyses, and which were not significantly different. Why did the authors not quantitate mRNA levels of Th2 markers such as IL-4 and IL-13? It would be interesting to see if overexpression of PRDX4 in the Tg mice increases their in murine lungs expression compared with WT mice, as the levels of TGF-b mRNA in lung homogenates in these mice are similar. This would help to address the question as to how PRDX4 worsens lung fibrosis when overexpressed in the bleomycin model.

Response: We measured the IL-4, IL-13 and IFN-γ mRNA levels in lung homogenates (Figure 7), and Tg-BLM mice showed a significantly lower mRNA expression of IL-4 and IFN-γ than WT-BLM mice, while Tg-saline mice had significantly higher IL-13 mRNA levels than WT-saline mice. In addition, we apologize for our incorrect description; “serum proinflammatory cytokines” on page 22, line 394 should be “proinflammatory cytokines in murine lung homogenates”. We have now revised our manuscript as follows:

Before

Page 14, line 237:

Total RNA extracted from the homogenized right lung tissue using ISOGEN reagent (Nippon Gene, Tokyo, Japan) reverse transcribed. CC chemokine ligand 2 (CCL-2), collagen I, connective tissue growth factor (CTGF), human PRDX4, interleukin (IL)-1β, IL-6, IL-17A, tumor necrosis factor (TNF)-α, platelet-derived growth factor subunit B (PDGF-B), active tissue growth factor-β1 (TGF-β1), fibronectin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expressions were quantified using real-time quantitative PCR using the ABI prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) as previously described [17, 25].
The total RNA extracted from the homogenized right lung tissue using ISOGEN reagent (Nippon Gene, Tokyo, Japan) was reverse-transcribed. The expression of CC chemokine ligand 2 (CCL-2), collagen I, connective tissue growth factor (CTGF), human PRDX4, interferon γ (IFN-γ), interleukin (IL)-1β, IL-4, IL-6, IL-13, IL-17A, tumor necrosis factor (TNF)-α, platelet-derived growth factor subunit B (PDGF-B), active tissue growth factor-β1 (TGF-β1), fibronectin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified using real-time quantitative PCR with the ABI prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA), as previously described [20, 26].

No significant differences were observed in IL-1β, IL-6, TNF-α, CCL-2, IL-17A, TGF-β1, CTGF, PDGF-B, and collagen 1β expression levels between WT-saline and Tg-saline; CCL-2 and IL-17A expressions in Tg-BLM were significantly higher than those in WT-BLM. However, no significant differences were found regarding other assessed cytokines and growth or profibrotic factors between these two groups (Figure 7).

The IL-13 mRNA expression in the lung was significantly higher in Tg-saline than in WT-saline mice, but no marked differences were noted in the IL-1β, IL-6, TNF-α, CCL-2, IL-17A, TGF-β1, CTGF, PDGF-B or collagen 1β expression. In Tg-BLM mice, the pulmonary mRNA levels of CCL-2 and IL-17 were significantly higher while those of IL-4 and IFN-γ were significantly lower than in WT-BLM mice. However, no significant differences were found in other assessed cytokines or in growth or profibrotic factors between these two groups (Figure 7).

In the present study, the level of serum proinflammatory cytokines such as IL-1β, IL-6, and TNF-α showed no significant differences between WT- and Tg-BLM.
In the present study, the mRNA levels of proinflammatory cytokines, such as IL-1β, IL-6 and TNF-α, in murine lung homogenates showed no significant differences between WT- and Tg-BLM.

(7) In this study, overexpression of PRDX4 in Tg mice drives pulmonary fibrosis in the bleomycin model. In contrast, in Ref 18 of the MS, pulmonary fibrosis is worse in PRDX1-knockout mice compared with WT mice. The authors should comment on this in the Discussion section.

Response: As described in line 394-428, PRDX1, one of the PRDX families, has been reported to have a protective effect as an antioxidant enzyme in the lung of IPF patients; however, as a conflicting effect, extracellularly released PRDXs, such as PRDX1, PRDX2, PRDX5 and PRDX6, have also been reported to cause severe inflammation as a danger signal (Ref 38). Our results suggest that the overexpression of PRDX4 in the lung may exert an exacerbating effect on pulmonary fibrosis by inducing inflammatory cytokines as danger signals rather than a protective effect as an antioxidant enzyme in the acute to subacute phase of pulmonary inflammation. We have now revised the manuscript as follows:
Conversely, extracellular PRDXs result in severe inflammation in the brain by functioning as danger signals in brain injury models [37]; thus, conflicting actions of PRDX4 have been reported in several inflammatory diseases.

Conversely, extracellular PRDXs, such as PRDX1, PRDX2, PRDX5 and PRDX6, induce severe inflammation in the brain by functioning as danger signals in brain injury models [38]; thus, conflicting actions of PRDXs have been reported in several inflammatory diseases.

Our results suggest that PRDX4 mediates danger signals, including the release of inflammatory cytokines, instead of inducing an antioxidant effect in the lungs; however, further investigation regarding this is necessary.

Our results suggest that the overexpression of PRDX4 in the lung may exert an exacerbating effect on pulmonary fibrosis by inducing inflammatory cytokines as danger signals rather than a protective effect as an antioxidant enzyme in the acute to subacute phase of pulmonary inflammation; however, further investigation regarding this is necessary.