Author's response to reviews

Title: Neutrophil Necrosis and Airway Inflammation in Lung Transplant Recipients with Cystic Fibrosis

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Cover Letter

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Article title: Neutrophil Necrosis and Airway Inflammation in Lung Transplant Recipients with Cystic Fibrosis
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To The BioMed Central Editorial Team:

We truly appreciate the reviewers' comments and suggestions. The suggestions were very helpful in improving the quality of the manuscript. We have addressed the reviewers' comments and suggestions as follows and revised the manuscript accordingly. The revised areas are highlighted in blue.

2135420382711070_Reviewer 1's report:

This study by Tsao and colleagues investigates the survival of neutrophils in cystic fibrosis lung transplant recipients with bacterial tracheobronchitis and suggests cleavage of annexin 1 as a marker of neutrophil necrosis. The study is of interest and the overall study design and data collection are straightforward and sound. However, I have some issues with the presentation and interpretation of the results.

Compulsory Revisions

1: My biggest issue overall is the interpretation of the results. According to the authors, annexin V positive cells are necrotic, however, most studies using this method conclude that annexin V positive cells are apoptotic. This is important as antimicrobial proteins are released in an unregulated manner during necrosis, but remain contained during apoptosis as described by the authors in the Background section. In addition, in the legend of Figure 4B the authors describe that the positive annexin V staining observed is a sign of apoptosis, however, in the results section the positive staining is interpreted as showing necrotic cells and is therefore confusing. In addition, the authors state necrosis as the fate of neutrophils in the infected airways in the abstract, but in the abstract, background and discussion they say oncotic, apoptotic and/or necrotic.

- Page 3 line 4 - Changed word "oncosis" to "necrosis".
- Page 12 last sentence of "Results" - Changed word "oncosis" to "necrosis".
- Page 15 second paragraph line 3 - Changed phrase "by both annexin V-FITC and PI" to "by PI".
- Page 15 second paragraph line 5 - Changed phrase "Rather, double labeling of cells with annexin V-FITC and PI" to "Rapid labeling of PI".
- Page 15 line 4 - Changed word "oncosis" to "impaired apoptosis".
- Page 15 line 5 - Changed phrase "how neutrophil activation and initiation of apoptotic and/or necrotic pathways in circulating neutrophils" to "how activation..."
and initiation of apoptosis in circulating neutrophils”.

2: When the authors state that annexin 1 degradation, which according to their conclusions is associated with necrosis, may begin in the inflamed airways, it would be beneficial to discuss potential differences in function of healthy and CF neutrophils that could cause the observed variation. The authors should especially be aware of a publication by Moriceau et al. (2010) that illustrates impaired apoptosis of CF neutrophils and heterozygous carriers without inflammation.

- We have cited the paper of Moriceau et al. in the discussion section (p.14 reference (21)). Thank you. Additionally, we have mentioned our unpublished observations concerning peripheral blood neutrophil apoptosis and BAL BPI levels in non-transplanted patients with CF (Discussion p.14).

3: Overall, the sample size used was quite small which makes the outcome of this study less robust and leaves room for subjective interpretation. Especially the Western blot results are only shown as images and were not quantified by densitometry which would give a more reliable way to compare samples. Additionally, for some experiments no number of repeats is given e.g. was flow cytometry only performed once or are results shown as representatives of n separate experiments (information of n should be included in the figure legends)?

- Respectfully, we agree with the reviewer that quantitative analysis of a large number of samples would be robust. However, we feel that the absence and/or degradation of annexin 1 to 33 kDa A1-BP are unequivocal markers to show the quality change of patient’s neutrophils.
- Figure 4 legend - As suggested by the reviewer, we have added the statement of "The results are representative of 10 experiments" in Figure 4 legend and "The results are representative of 8 experiments" in the Figure 5 legend.

4: I would like to see a comparison of neutrophil viability of healthy and CF circulating neutrophils. The results from the flow cytometry analyses are designed to link protein degradation and necrosis, however, they were only performed for healthy circulating neutrophils. It is possible that protein degradation could be caused by something completely unrelated to necrosis e.g. something that is characteristic to CF (inflammation, etc...). Therefore, I recommend the addition of flow cytometry data for CF blood neutrophils to support the suggested conclusions.

- The reviewer has offered valuable suggestions. However, due to the unavailability of samples, we are not able to perform the suggested flow cytometry analysis of CF blood neutrophils. We will strongly consider the reviewer’s suggestions in future studies. However, as mentioned under #2 above, we have mentioned our unpublished observations concerning peripheral blood neutrophil apoptosis and BAL BPI levels in non-transplanted patients with CF.

Minor Essential Revisions
1: Change normal volunteers to healthy volunteers.
   - Page 2 Results line 6 - Change word "normal" to "healthy".
2: Remove commas before “and” as they are not needed.
   - We have removed commas before “and”.
3: The legends of Figure 1 and 4 should include some information of the imaging methodology used (e.g. light microscopy, original magnification, staining (with trypan blue))
   - Imaging methodology is now described in the legends of Figures 1 and 4.
4: It would be clearer for the reader if all abbreviations used in the figures are described in the figure legends, such as HS (healthy subject) and LTx (lung transplant recipient).
   - All abbreviations used in the figures are now described in the legends.
5: Abstract Methods: remove via “...was determined by morphologic appearance...”
   - We deleted “via”.
6: Abstract Results: insert comma after “...particularly to a 33 kDa annexin 1 breakdown product (A1-BP)"
   - Comma was inserted.
7: Abstract Conclusions: remove comma after tracheobronchitis
   - Comma was removed.
8: Background: remove comma after neutrophil-dominated (line 2)
   - Comma was removed.
9: Background: remove comma after “neutrophils undergo necrosis” (line 12)
   - Comma was removed.
10: Methods Isolation of BAL: explain abbreviation BAL and revise sentence in line 1
    - We added explanation of abbreviation BAL in Introduction when it was first mentioned (p.3 last sentence).
11: Bronchoalveolar lavage (BAL) fluids (BALF) were obtained from healthy (instead of normal) volunteers, patients with CF (remove comma) and clinically...
    - We revised the words accordingly.
12: Methods Isolation of BAL, line 12: rephrase and change “BALF cultures of BALF” to “bacterial (or microbial) cultures of BALF”
    - “BALF cultures of BALF” was changed to “bacterial cultures of BALF”.
13: Methods Isolation of BAL, section 2, line 4: remove full stop after 10min
    - Full stop was removed.
14: Methods Isolation of BAL, section 2, line 7: remove comma after cytospin slide preparation
    - Comma was removed.
15: Methods Isolation of BAL, section 2, line 8: remove triple space between full stop and “The rest of the cell...”
    - Triple space was corrected.
16: Methods Isolation of BAL, last line: remove comma after “...before use...”
    - Comma was removed.
17: Methods Cell culture: rephrase “About” to “On average 3x10^7 neutrophils were isolated from...”
    - “About” was changed to “On average...”
18: Discussion, section 2, line 3: names of bacteria in italics and spell full name as abbreviation has not been explained elsewhere before
    - Full names of bacteria are giving in italics.
19: Figure legend 1: rephrase to read “Morphological analysis of neutrophils
isolated from peripheral blood from a healthy subject (HS PB) and a lung transplant recipient (LTx PB)."

- The phrase has been changed as suggested.

20: Figure legend 2: remove underlining of "BAL cells and peripheral cells"
- Underline was removed.

21: Figure legend 2: remove “Right middle and bottom, same samples of Left meddle and bottom”, instead extent sentence in line 4: “Left middle and bottom (remove comma) lanes of HS1-HS3"
- Figure 2 was re-arranged as suggested and the legend was re-phrased.

22: Figure legend 3: line 3 capital W for Western blot analysis
- Changed.

23: Figure 5B: remove typo in the label “control"
- We deleted “control”.

Discretionary Revisions

Figure 2 would benefit from rearranging some of the panels. It would be clearer for the reader if for example all neutrophil related Western blot panels appeared on the left with the actin control directly below the annexin 1 blot of peripheral cells and accordingly for macrophages on the right.
- We have rearranged Figure 2 as suggested.

It would be interesting to know how many lung transplant recipients actually develop purulent bacterial tracheobronchitis (is it a common risk?) and if there was a difference between CF and other lung transplant recipients. So if this data is available it should be included for example in the discussion.
- We have added our comments on this issue to the discussion. We have frequently observed purulent bacterial tracheobronchitis with benign BAL cell profile and pathogen analysis at surveillance bronchoscopy in lung transplant recipients with CF, but this is rare in non-CF transplant recipients.

**Level of interest:** An article of importance in its field

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:**

I declare that I have no competing interests

8882955957110717_Reviewer 2's report:

This is a largely observational study concerning the expression of Annexin-I in neutrophils and macrophages from CF patients, CF patients following lung transplant and healthy controls. The authors show that annexin-I is degraded not only in obviously
necrotic PMNs from the airway of post lung transplant CF patients, but also in the circulation. Further experiments are performed showing that this degradation proceeds as cells move beyond apoptosis to necrosis and also in response to PMA. On the basis of this, the authors suggest that 'Annexin-I degradation to 33kDa A1-BP can be used as a sensitive marker to detect neutrophil oncosis'. The concept of a sensitive peripheral marker of airway inflammation is a useful and interesting one. However, whilst the experiments as performed and the evidence as presented in the manuscript can support this view, data are not presented from a sufficient number of subjects to be confident about this statement.

Major Compulsory Revisions:
1) There is no indication of how many patients have been enrolled into this study.
   - We have observed proximal airway inflammation & infection without peripheral (BAL) neutrophilia or significant bacterial CFU in a total of 14 lung transplant recipients, and neutrophils and annexin 1 degradation were examined in depth in 6 recipients. This has now been stated in the manuscript.

2) It has not been stated whether each presented figure represents a standalone experiment or is representative of a number of independent experiments.
   - The results are representative of a number of independent experiments. We have described the number of experiments in the legends.

3) There is no statistical analysis of the data.
   - Respectfully, we agree with the reviewer that quantitative analysis of a large number of samples would be robust. However, we feel that the absence and/or degradation of annexin 1 to 33 kDa A1-BP are unequivocal markers to show the quality change of patient's neutrophils. As we described in the Discussion section, the quality change of neutrophil annexin 1 is more evident than quantitative analysis of the decrease actin content.

4) Data presented in figure 2 suggest that those neutrophil lysates lacking annexin-I are also deficient in Actin. This suggests that a generalised degradation of cellular proteins may have occurred. Do the authors have evidence that other proteins are intact in these lysates? This would support the idea that Annexin-1 is a specific marker of airway inflammation.
   - We did the analysis of neutrophil proteins of healthy subjects and patients by gel electrophoresis and staining. The results suggested a generalized degradation of cellular proteins. Because gel electrophoresis staining contained total cellular proteins, it was difficult to show the significance. Therefore, annexin 1 and actin were selected as representative proteins. Although annexin 1 is not the only protein degraded, its degradation to form a 33 kDa breakdown product is highly sensitive to show the onset of intracellular protein degradation in neutrophils.

Minor Essential Revisions:
5) Annotation in figure 5 should read ‘PMA’ not ‘PMN’.
   - We have corrected the label. Thank you.
6) ’injure’ to ‘injur’ in background of Abstract.
   • The word “injure” is correct.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests
____________________________________________________

5200420347153455_Reviewer 3's report:

Overall this was a very interesting article and a nice addition to the authors’ previous work on annexin 1. They address the status of neutrophils in post lung transplant tracheobronchitis and present data consistent with altered neutrophil morphology, increased response to PMA stimulus, and show an increased presence of degraded annexin 1, presumably with loss of the anti-inflammatory N terminus. The work is limited by an absence of any data regarding patient drug regimen, and the authors do not address if the increased priming of circulating PMNs for necrosis could in any way be due to treatment. The authors can easily address this with a statement that the biological effects of treatments are either unknown or being considered. All other issues are minor, primarily editorial in nature. The title should be made more specific incorporating annexin 1 as this seems to be the main protein degradation product shown to be affected. There is a font change in the method section- subsections entitled “isolation of neutrophils and monocytes from peripheral blood and cell culture of neutrophils” appear to have different font or line spacing than the remaining manuscript. There are some spelling errors. In Figure 2 legend “meddle” should be middle. On Figure 5 “Ccontrol” should be control. In addition, for figure 4 and figure 5 some labeling was lost on printing due to the size of figure. The methods are well documented and appropriate for the work presented. In figure 1 the image shown for the neutrophils of CF BALF does not seem to represent the conclusions drawn in results p.9 and appears to be more like that of the LTX BR Asp than HS. The authors may consider a clearer image for that panel. Further work delineating the pathways leading the appearance of this early stage of neutrophil necrosis in the circulation and the effect on localized tissue inflammation, would broaden the scope of our understanding of post transplant disease processes and could potentially lead to new drug targets for inflammation, especially if the systemic effects are a result of a yet unidentified viral or fungal toxin.

   • We comment on the possibility the immunosuppressive or other medication that were taken by the lung transplant recipients may have affected neutrophil behavior in the revised manuscript.
• In the revised manuscript, the title has been changed by including "Annexin 1 Degradation".
• The font and line spacing have been corrected.
• Spelling errors in Figure 2 legend and Figure 5 have been corrected. Thank you.
• Figures have been resized to ensure full figures on printing.
• We have clarified the description of the results of Figure 1 on p.9.
• We have increased the resolution of the figure. Regretfully, we currently could not provide images better than the ones shown in the panels.
• We fully agree with the reviewer’s view of the importance of further work delineating the pathways leading the appearance of the early stage of neutrophil necrosis in the circulation. Analysis of annexin 1 degradation may be a useful tool for such studies.

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:** 'I declare that I have no competing interests'