Author's response to reviews

Title: Monocyte unresponsiveness and impaired IL1beta, TNFalpha and IL7 production are associated with a poor outcome in Malawian adults with pulmonary tuberculosis

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Author's response to reviews: see over
Dear Editors,

Thank you very much for the helpful and constructive comments on the paper. Below I have made my point-by-point response to the comments and suggestions. The revised manuscript with changes tracked is also uploaded.

1.1 The objectives should be more clearly defined in terms of the authors’ ambitions. As it stands, it seems like objective 1 is merely descriptive, whereas the predictive potential of their findings has considerable relevance to the management of TB patients through the identification of patients who would benefit from closer follow-up and supportive therapy. This aspect is not raised or discussed and would clearly increase the clinical relevance.

Response: The aims have been amended to indicate that HLA-DR expression in sepsis has been suggested as a prognostic biomarker, and that ‘we sought to determine whether patients at high risk of clinical deterioration could be identified from a specific biomarker profile’. Whilst this aim was not fully achieved (as discussed later), it was indeed our desired outcome.

1.2 A study flow-chart depicting the subgroups included in the study in relation to 1) the mother study and 2) the performed lab assays, sample time points and results is required to get a clear picture on how the study is conducted. Unclear contents are specified below. If understood correctly, the analysis in this study is based on 2 distinct subgroups of patients included in the 321 Malawian TV cohort which are addressed separately with regard to revision requirements:

2. The first subgroup includes 22 patients with ‘poor outcome’ defined as death on life-threatening clinical deterioration during the first 2 months of TB treatment.

2.1

i) It is not clear whether these 22 constitutes all patients with poor outcome or whether these had been subject to selection. This must be clarified and if a selection was made, an explanation of the selection process must be added.

Response: A flow diagram has been added to indicate the processes. These were indeed all the patients who died or suffered a life-threatening event necessitating admission to hospital. No selection was therefore applied. This has been emphasised in the methods.

ii) I would have liked to see a table depicting basic clinical characteristics like age and gender, death or life threatening event, HIV status and BMI, stratified by clinical group. The selection of matched controls is sufficiently explained.

Response: Table 1 has been added to summarise these criteria, in addition to the nature and day of the acute illness or the clinically attributed cause of death. The methods for determining the causes have not been fully discussed in this paper as they were detailed in the JID publication and I did not want to bring too much repetition.
iii) Cytokine analyses (17-plex) in unstimulated (serum) and stimulated whole blood supernatants were performed in all 44 patients prior to initiation of TB treatment (Day 0) and after 7 days. It is not stated in the manuscript, but from Waitt et al JID 2011, I assume that cytokine levels in unstimulated serum samples were subtracted from the levels in the stimulated samples. This should be made clear in the current manuscript. I would also appreciate a brief description of the WB assay, at a minimum by mentioning the stimulants and stimulation time used. AUC for cytokine production between day 0 and 7 of TB treatment is compared between the groups. The choice of this measurement is not evident and therefore must be explained.

Responses:

1) The whole blood assay had been described in more detail in the ‘mother paper’, however for clarity the method has now been summarised here too (Lines 1-7 of methods)

2) The unstimulated value was indeed subtracted from the stimulated value, and this detail has been added to the text (Line 6-7, page 5)

3) The methods have been expanded to indicate the stimulants and time of stimulation (lines 4 and 5, page 5)

4) The rationale for choosing the AUC from Day 0 to Day 7 for cytokine concentrations was chosen to provide a measure of cytokine exposure during that time. This clarification has been added to the text (Lines 14-15, page 5)

iv) Only 6 patients (and their matched controls) were included in the analyses of cytokine dynamics through the intensive phase of TB treatment. These all recovered. The low number is not explained. Did the rest of the 22 patients die? This can be depicted by the flow chart as previously suggested. The only time points measured to assess cytokine dynamics seem to be day 0 and 56. By definition, the deterioration took place during the first 2 months of treatment, but within the time frame there could be considerable variation in timing of the onset of deterioration. This is not discussed.

According to the mother study, the patients were reviewed per protocol on days 3, 7, 28 and 56 and had an additional assessment if admitted with deterioration. With regard to the referrals to reduced monocyte function in bacterial sepsis, it would have been very interesting to add to the longitudinal figure (fig 2) the cytokine measurements at admission for deterioration. The choice of Day 0 would be relevant if assessed for their potential prognostic markers of later deterioration on treatment, but this applicability is not mentioned or discussed. The author might choose to increase the resolution on the lower parts of the y-axes displaying cytokine concentrations in Figure 2. Knowing which of the cases who had HIV co-infection is of interest even though this characteristic was subject to matching in the statistical analysis.

Response:

i) The reason for the inclusion of only six patients and their controls was that overall 10 patients recovered from their deterioration and 12 died (Revised Figure 1 indicates this). Of the 10 who recovered, there were six patients who had matched Day 0 and 56 samples. These time points were chosen for the luminex analysis as the objective was to look for immunological recovery and this was the latest time point included in the study (Lines 18 and 19, page 5). I agree it would be interesting to have tracked these levels in more detail through the time of deterioration, but these results are not available (see response below)
ii) The timing of clinical deterioration has been added to the new Table 1

iii) The HIV status (and CD4 count) of the cases and controls has been added to the new table (Table 1)

iv) Cytokine levels at day 0 alone were not found to be of prognostic significance in terms of predicting clinical deterioration. A statement to this effect has been added to the results section

v) The cytokine profiles from patients at the time of deterioration are not available

vi) The resolution of the titles and axes for Figure 2 has been increased

3. The second subgroup of TB patients (used to address objective 2) consists of subjects in ‘a further cohort of 30 consecutive patients enrolled in the overall study’.

3.1 I don’t understand what is meant by the cited explanation of the 30 patients above. Was this unrelated to outcome? Samples from these patients were used for monocyte immunophenotyping and ICS. The methods are adequately described but ‘unstimulated’ or ‘stimulated’ should be added before whole blood on page 5 line 9 for clarity. It is also unclear whether a minimum of 100 000 events refer to events within the monocyte gate or all PBMCs. Suggested presentation of TLR data in a table.

Response:

i) The selection of these patients was limited due to the necessity of performing the assays in real time rather than being able to use stored samples. Therefore the selection of these patients was not related to outcome, but rather a ‘convenience sample’ of the final 30 patients included in the study. The preliminary results (described fully in the mother paper) became available towards the end of the cohort (and actually were the opposite of the initial primary hypothesis which anticipated and exaggerated cytokine response among the poor outcome group), and therefore this was the only way to be able to incorporate these assays. We accept this as a limitation and have therefore commented specifically on this in the limitations section. I have amended the description of the cohort to clarify that these assays were done in ‘real time’ in a separate cohort to the case-control study, and have added Figure 1 to further clarify the relationship of the populations

ii) Stimulated has been added to the method

iii) 100 000 events referred to all PBMCs; this has been added for clarity

iv) A revised Table of results has been added.

4. Statistical Concerns

4.1 In the description of the statistical methods, the authors state that correction for multiple testing (11 cytokines) was done by adjusting the p value for significance to 0.0045, but the applied method, Bonferroni correction (misspelled) is stated only in Supplementary Table 1 legend and not in the main manuscript. Strangely, all the listed p-values for differences in cytokine levels written to be
significant findings are >0.0045. I might have misunderstood this, but then the explanation given is too poor....

Response:

i) The spelling of Bonferroni has been corrected, and added to the methods

ii) Indeed, all p values are >0.0045 and therefore in the results section we have not used the phrase ‘significantly’ but instead have referred to trends. I had used the term ‘considerably lower’ in the first line of the results, but have removed this ambiguous term and have emphasised that we have shown trends. The conclusions were tempered to indicate these are trends which require follow-up and validation in a future cohort (existing text highlighted yellow).

4.2 Supplementary table 1- median and IQR for 11 cytokines with CV <15% is given, but then the p-value is according to the legend, based on T-tests.

Response: This is an error, and the p-values were indeed corrected using the Mann-Whitney test for non-parametric data. I have highlighted the section where I have stated this in the methods, and corrected the table legend. (Supplementary table_R1)

4.3 Should an adjusted p-value (Bonferroni correction) apply for the longitudinal data presented in Figure 2?

Response: Figure 2 illustrates trends which do not reach statistical significance, and the sample size was too small to draw firm conclusions from the data. This has been stated and acknowledged as a limitation. The term significant has been removed from the description of these data and replaced with ‘trends’

5. The term classical CD14hi16lo monocytes should be explained

Response: This has been clarified

Page 9, line 22 ‘in TB’ should be added for clarity

Response: This has been corrected

I hope that the revised manuscript is now acceptable.

Yours sincerely,

Catriona Waitt