Title: Cytokine response to selected MTB antigens in Ghanaian TB patients, before and at 2 weeks of anti-TB therapy is characterized by high expression of IFN-gamma and Granzyme B and inter-individual variation

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Author's response to reviews: see over
RESPONSE TO REVIEWERS

Reviewer 1: Norman Nausch

General comments

1. The main problem of this study is the inconsistent statistical approach used by the authors. Authors switch between parametric and non-parametric tests, while the justification to use parametric tests is fragile and needs to be clarified.

Response: We would like to thank the reviewer for this helpful comment and have only maintained the statistical analysis using non-parametric tests. This in no way alters the outcome of the study.

2. In addition Table 3 (T-Test), Figure 2 and 3 basically address the same question in different ways.

Response: Figure 2 was based on the output of the ANOVA test for effect of time point on cytokine secretion. We have opted to use the Mann-Whitney test which looks at the same issue in a different way by comparing the cytokine profile at baseline and after week 2 of treatment so Figure 2 has been removed. Table 3 has however been maintained because it gives the detailed breakdown of the levels of cytokines produced and the number of samples analyzed while Figure 3 gives the general trend in terms of changes at week 2.

A. Abstract

1. Specify/Clarify aim of the study. Reading the background it sounds like authors investigate predictors of treatment outcome. However, finally they only analyze if cytokine responses improve 2 weeks after treatment and thus might have the potential to provide a tool for early prediction. That should be clarified/rephrased.

Response: Thanks for this observation. We have rewritten the background to make it clear that the primary objective was to determine the nature of the immune response to Mtb specific antigens during the first 2 weeks when it is thought that TB patients are no longer infectious.

2. Conclusion: However, due to the wide inter-individual variation observed, a wider pool of cytokines and Mtb specific antigens would have to be investigated to discover the most effective combination for monitoring treatment response’. Is that statement necessarily true? Maybe rephrase.

Response: This conclusion has been rewritten to address the specific aim of the study.

B. METHODS
1. Ethical approval should be moved to the top of Method section and include the consent statement.

Response: The methods section now begins with a statement on Ethical approval and consent. Page 5, Lines 88-92

2. PBMC culture: Nunc plate 163320 seems to be a 96 well plate with a volume of 0.3ml, but the authors state that 0.5ml/well was used. Please clarify.

Response: This was an error, 48 well -plate (Nunc Cat No.152640) was used. This has been corrected. Page 8, line 145

3. It is stated that one HIV+ participant was excluded from the total of 20 participants, but Table 3 and Figure 1 still contains 20 participants. Please ensure that the HIV+ participant is excluded throughout the manuscript.

Response: This has been corrected

4. One way Analysis of Variance (ANOVA) was performed for the effect of time point on mean cytokine concentration (Unmatched analysis). Please specify why authors used an unmatched ANOVA for the effect of time point and not a repeated measures ANOVA? In addition, it is questionable to use a parametric ANOVA instead of a non-parametric test here, whereas for some other tests non-parametric test were used. The same for the T-Test used in Table 3.

Response: This has been addressed based on the general comments of reviewer 1. The parametric ANOVA has been removed so the analysis only includes the non parametric tests.

5. A uni-variate analysis on less than 20 samples is questionable.

Response: We are aware of this, however, despite the small sample size we needed to attempt to identify factors which would account for the change in 2 weeks.

C. RESULTS

1. MAF is not spelled out

Response: MAF (Mycobacterium africanum) has now been spelt out. Page 12, line 235

2. Figure 1 might not be required or moved to supplemental data (optional)
Response: We have opted to maintain Figure 1 as it shows the stimulation index.

3. Figure 2: In the legend, it is stated ‘The majority of trends are not statistically significant, however, due partially to small sample size’. Nevertheless, data were transformed to satisfy assumption of normal distribution? This sounds precarious. Please indicate the results of normal distribution and clarify this approach. In 3.5 a Wilcoxon paired test was used, which, in the reviewer’s opinion, is the most valid test here.

Response: This is also in reference to the ANOVA test used which has now been removed.

4. The idea to use a uni-variate analysis to test the influence of sputum smear result and age is reasonable, however with a sample size of 20 questionable.

Response: We are aware of this, however, despite the small sample size we needed to attempt to identify factors which would account for the change in 2 weeks.

D) Discussion:

Table 1 is in a bad quality and table numbers should be in the order they are mentioned in the text.

Response: Table 1 (Antigens used in the study and their protein size) has been removed from the manuscript and the information incorporated into the methods sections. Page 6, Line 110-113 Table numbers have been re-arranged to reflect the order they are mentioned in the text.

REVIEWER 2: Simani Gaseitsiwe

Major Compulsory Revisions

1. As the study wants to use Mtb specific cytokines as predictors of treatment outcome, would it not be better to use a cohort in which the treatment outcomes vary rather than the current cohort where the treatment outcome is the same at 2 months (pg 10 line 187-188 All except one subject converted to a negative sputum smear at 2 months of treatment)? If the treatment outcome is the same (I fail to see any other measure of treatment outcome in the whole manuscript besides the sputum smears at 2 months) why should we expect the cytokines measured to differ?

Response: The study sought to determine the nature of the response at 2 weeks when it is thought that most of the actively replicating bacteria are killed and thus determine the utility of Mtb-specific cytokines during this period as predictors of early treatment response. There is currently no evidence of correlation between sputum conversion at two months and specific changes in the immunological profile at week two of treatment. We did not expect changes in cytokine profile by two weeks of treatment to differ considerably in this cohort of patients. We
tried to ascertain if this similar cytokine profile could be utilized as an early treatment response. We were unable to determine smear status at week 2 which would have allowed us to correlate the immunological response to microbiological improvement. However, we have rewritten the background to make it clear that the focus of this article is on determining early changes in cytokine response. Though it would have been interesting to assess differences in early cytokine responses among patients who were successfully treated and those who had treatment failure, our cohort happened to consist predominantly of patients who responded well to treatment.

2. Despite having access to PBMCs, the authors decided to focus only on the cytokine released and not on the phenotypes of the cells that release the cytokines. It is not clear why this was the case whilst it is clear from the literature that the phenotype of the cells releasing the cytokines is just as important in determining the quality of the immune response to an antigen. The authors need to justify if their choice of experiments were best suited to address this important question more so that they do not mention this as a limitation.

**Response:** We are aware that the phenotypes of the cells releasing the cytokines are very important. We actually determined the phenotype (CD4 and CD8) and we have now included those results in this manuscript.

3. It would be important to mention in the manuscript what motivated the selection of the 6 cytokines that were measured.

**Response:** We have mentioned this now, in the introduction Page 5, line 71-73

**Minor Essential Revisions**

1. Pg 14 Line 266, do away with the second "to"
2. Pg 14 line 272, do away with "is"
3. Pg 15 line 278, should be … CD8+ T cells
4. Table 1, Font and style should be improved

**Response:** Changes have been effected as follows and table 1 font has been changed to 12