Author's response to reviews

Title: Expression of M. tuberculosis-induced Suppressor of Cytokine Signaling (SOCS) 1, SOCS3, FoxP3 and secretion of IL-6 associates with differing clinical severity of tuberculosis

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Author's response to reviews: see over
Dear Dr. Thwaites,

Thank you for providing us the opportunity to revise our paper for resubmission to BMC Infectious Diseases. We have tried to address each of the concerns raised by the authors and provided point-by-point answers to each query.

I hope that you will now find the revised manuscript satisfactory for publication in your journal.

With best wishes

Zahra Hasan, PhD
ANSWERS TO REVIEWERS COMMENTS

Reviewer 1

Major

1. A modest number of candidate genes is investigated – inevitably the amount of information acquired is limited in comparison with recent studies that take a genome-wide approach to identifying genes whose expression differs between patients with disease at different sites vs controls.

Answer:

Thank you for this comment. The Reviewer is correct in stating that we have only studied a small subset of genes in comparison to other studies which have used microarray analysis to perform genome-wide association studies. However, we have focused on a different aspect of TB immunology in this study as compared with those which have looked at bio signatures of active TB [1], gene profiles which possibly characterize latent TB [2] or gene profiles which are modified with treatment of TB [3]. The utility of IFN-γ pathway genes in identification of TB disease signatures is highlighted in a number of recent studies [4]. However, these studies have not investigated an association of gene expression profiles with clinical disease severity of TB at different sites, and this is the objective of our study. Hence, we believe that albeit we have looked at the expression of a limited number of genes there is value in the association that we draw between them. In this revised manuscript we have provided a more comprehensive discussion and highlighted the novel aspects of this study.

2. Study participants are categorised into many different sub-groups, each of them small – consequently it is hard to interpret the numerous associations reported. This sub-division also introduces potential for type I error to creep in – but no adjustment has been made for multiple analyses. Either the number of sub-group analyses should be reduced, or an adjustment for multiple analyses should be applied.

Answer:

We thank the reviewer for pointing out a possible error in statistical analysis of the sub-groups of TB patients. To address this we have taken advice from a statistician and performed an additional analysis of the same data set. Previously we used Mann-Whitney U test to compared mRNA expression and cytokine secretion profiles between groups of TB patients as compared with ECs. We have now first applied Kruskal-Wallis non-parametric analysis to compare subgroups of TB patients, followed by Mann-Whitney U analysis to compare the groups within each data set where we have observed a significant difference in p values <0.05 as per the Kruskal-Wallis test.

In addition, what was previously numbered Table 3 and is now numbered Table 2 (where cytokine secretion profiles are described) the sub-groups have been merged into the larger PTB and ETB groups for more stringent statistical analysis.

3. The abstract concludes that expression of SOCS1 and SOCS3 impact on Th1/Th2 balance, which in turn determine outcome of infection – but to infer this sequence of cause and effect on the basis of cross-sectional associations is to go beyond the data. The differences in gene
expression patterns seen in patients with moderate vs severe disease could be a cause or a consequence of disease severity, for example.

Answer:

Following reviewer’s recommendations, we have improved the abstract by rewriting the conclusion

Minor

1. Title: I had to read this a few times to get my head around it – is it possible to simplify the title to convey the key message? One suggestion: Expression of M. tuberculosis-induced Suppressor of Cytokine signalling (SOCS) 1 and 3 and FoxP3 associates with clinical severity of tuberculosis

Answer:

As per reviewers suggestions we have modified the Title of the study to read ‘

Expression of M. tuberculosis-induced Suppressor of Cytokine Signaling (SOCS) 1, SOCS3, FoxP3 and secretion of IL-6 associates with differing clinical severity of tuberculosis

2. Abstract: Background: ‘IFN-gamma is lowered in tuberculosis (TB)’ – does this statement refer to circulating levels, antigen-stimulated levels, gene expression or other? Wording should make this explicit

Answer:

As per reviewer’s kind suggestion, this statement has been clarified as

‘IFN-γ stimulated responses are lowered in tuberculosis (TB), while expression of Suppressor of Cytokine Signaling (SOCS) molecules – 1 and 3 and CD4+CD25+FoxP3+ T regulatory cells is increased’

3. The abstract contains numerous unconventional abbreviations (e.g. L-ETB, D-ETB), which make it difficult to interpret the Results section readily

Answer:

We have reduced the used of abbreviations in the Abstract to make it easier to read.

4. Introduction: ‘CD4+ T cells play a central role in containment of M. tuberculosis infection by secreting Interferon-gamma (IFN-γ), IL12 and TNF’ – IL12 and TNF are primarily secreted by macrophages rather than CD4+ cells

Answer:
In the revised manuscript, we have corrected the above mentioned sentence:

‘CD4+ T cells play a central role in containment of *M. tuberculosis* infection by secreting Interferon-gamma (IFN-γ) [5]. Coordinated Tumor necrosis factor alpha (TNF-α), and Interleukin-12 (IL-12) secretion by macrophages and dendritic cells respectively have also been implicated in a protective immune response to *M. tuberculosis* [6]’. See page 4, para 2.

5. Methods – a single 18h time point was employed for assays of gene expression and cytokine secretion. However, antigen-stimulated IFN-gamma may not reach peak concentrations until 72h or later post-stimulation. Were timecourse experiments conducted to justify selection of this single timepoint? If not, then this needs to be noted as a study limitation.

Answer:

In the present study, the gene expressions and cytokine secretions were only measured at 18 hrs. Therefore, as per reviewers kind suggestions we have further clarified our results by stating that:

‘We measured all the cytokines simultaneously after 18 h of culture. This time point has been found to be suitable for measuring most cytokines [7] except IFN-γ responses which are increased in up to 6 days of culture [8, 9]’. Therefore it may be that we have underestimated the IFNγ levels in our study. See page 14, para 1.

6. *Table 1 seems to list characteristics of study participants rather than ‘diagnostic criteria’ – these should appear at the beginning of results, rather than in Methods as they do presently*

Answer:

As recommended by the reviewer, we have moved Table 1 from Methods section to the results section and changed to title to ‘Characteristics of study subjects’.
Reviewer 2

The results are interesting and support a dysregulation of host immune responses to TB infection.

The paper is not particularly well written (eg page 5 line one ‘...levels of down-modulatory IL10’) and the data is poorly presented (see below).

We thank the reviewer for his comments. We have revised the paper with the help of a native English language speaker so that it is easier to read and have tried to improve the presentation of the data as per the suggestions provided.

Minor revisions:

1. The authors need to state how many of the individuals in each of their disease groups had neither microscopy/AFB culture nor suggestive histology and therefore potentially did not have MTB infection.

Answer:

All the patients included in the study had a confirmed diagnosis for TB based on microscopy or culture or histology. Perhaps this was not clear from the previous version of the manuscript and we have written this more clearly in the Methods section and also by modifying Table 1, Characteristics of study participants.

2. Table 2 and 3 the tables should include the mean difference following stimulation and need to show the actual p value for each comparison as a separate column. The data should also be graphed (to make clear which results have been statistically compared. The individual patient data should also shown to see the differences in response to MTB exposure)

Answer:

We thank reviewer for his kind suggestions. In an effort to improve the data presentation we have now presented the data in Table 2 as a figure (Fig 1) which illustrates gene expression in un-stimulated and MTB stimulated PBMCs in patients and control groups. We performed Wilcoxon rank test analysis on data to compare the effect of MTB exposure on each set of samples. However, we found that in all of the cases studied there was no difference between unstimulated and M. tuberculosis stimulated gene expression levels in the case of IFN-γ, SOCS1, SOCS3 or FoxP3. This may be due to the already raised mRNA titers in TB patients as a consequence of endogenous stimulation in the M. tuberculosis infected host. This information has been included in the results section. See page 11, para2.

Table 3 included cytokine secretion data from PBMC cell supernatants pre and post-MTB stimulation. Following reviewer’s valuable suggestions we have now moved this up as Table 2 and merged the sub-groups of PTB; moderately advanced PTB (PTB-mod) and far advanced PTB (PTB-adv) and ETB; less severe ETB (L-ETB) and severe ETB (D-ETB). Mean differences between M. tuberculosis stimulated and un-stimulated cytokine secretion levels were also calculated and included as a separate column. Further, as per the suggestions of Reviewer 1,
additional statistical analysis have been performed and p values based on Kruskal-Wallis test are given in the table, while Mann Whitney U test analysis data was used for comparison of groups within each data set.

3. The authors must comment on how changes in the contribution of different leukocytes to the total PMBC number could influence cytokine/SOCS levels.

Have the patients had differential white cell counts at the time of PBMC?

Answer:

In the present study, All TB patients showed an increased total leukocyte count but decreased lymphocytes as compared with controls. Therefore, as per reviewers kind suggestions we have included this data as supplementary Table S1.

‘All TB patients showed an increased total leukocyte count but decreased lymphocyte counts as compared with the control group. The increased SOCS1 mRNA expression observed in these patients despite a reduced lymphocyte count could be attributed to SOCS1expression from macrophages in addition to the T cell compartment [11, 12]’. We have now included above mentioned points in the Discussion. See page 14, para 4.
References


