nature portfolio

# Peer Review File

## A Serum B-Lymphocyte Activation Signature is a Key Distinguishing Feature of the Immune Response in Sarcoidosis Compared to Tuberculosisce

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Putera et all report an interesting study of serum proteins comparing between sarcoidosis and TB patients, and with respective control groups. The study is timely as it remains a major clinical challenge to discriminate sarcoidosis from TB and specific protein biomarkers could be of major use. However there are some limitations inherent to the study design, which the authors have done their best to accommodate, yet this still raises questions about the results and their interpretation.

Given the age & sex differences present in sarcoidosis and TB patients how were these factors accounted for in the analysis? considering that such factors both have major effects on both immune responses and serum protein levels. At a minimum the comparative analysis between disease and controls could be corrected for age and sex.

Also the sample sizes are quite different between sarcoidosis and TB which will have a major impact on the differences observed, the numbers of differences, and strength of differences observed between the two disease groups. One analytical way to address this would be to report effect sizes for the different proteins which is also a better reflection of biological significance than p values. The authors need to acknowledge these limitations in their study design, which means there is likely validity in the commonly observed pathways but interpreting differences is much more challenging.

In line with this could the authors perform down sampling of the sarcoidosis cohort to match the TB cohort size specifically for comparing effect sizes in the B cell signature that they highlight is specific to sarcoidosis, this would give further confidence in the reported results.

Why does the last results section which is a major part of the manuscript only have a supplementary figure, especially when there are only 4 main figures ? However this section is not convincing for the reasons indicated above.

Minor

Figures are not numbered in the PDF which is not helpful but I guess this is a journal formatting issue.

Discussion line 449 typo “the immunostaining staining”.

There is no data availability statement in the manuscript which should indicate where the data sets will be deposited to allow other researchers to reuse the data.

Reviewer #2 (Remarks to the Author):

The results of this manuscript underscore the significant clinical overlap between sarcoidosis and tuberculosis. However, we are reminded that other infections may mimic sarcoidosis, such as syphilis.

To optimally exclude patients with alternative reasons for uveitis, the authors should clearly state how many patients (sarcoidosis, TB) had a "complete" workup with ocular fluid analysis for infectious pathogens and blood tests to screen for TB, syphilis, and autoimmunity.

Otherwise, the study results seem to be consistent with prior reports and identify potential therapeutic targets. As stated by the authors, a larger study will be needed to verify these results.

July 4th, 2024

Dear Editors,

Thank you for giving us the opportunity to resubmit a revised version of our manuscript entitled " A serum B-lymphocyte activation signature is a key distinguishing feature of the immune response in sarcoidosis compared to tuberculosis" to Communications Biology. We really appreciate the insightful comments from the reviewers; they've been instrumental in improving the scientific content of our manuscript. We have carefully revised our previously submitted manuscript, addressing each point raised by the reviewers. Once again, we would like to thank the editors and reviewers for their thoughtful consideration of our submission. We hope that our revised version meets the standards for publication in this esteemed journal.

**Reviewers' comments:**

Reviewer #1 (Remarks to the Author):

1. Putera et all report an interesting study of serum proteins comparing between sarcoidosis and TB patients, and with respective control groups. The study is timely as it remains a major clinical challenge to discriminate sarcoidosis from TB and specific protein biomarkers could be of major use. However there are some limitations inherent to the study design, which the authors have done their best to accommodate, yet this still raises questions about the results and their interpretation.

- **Answer:** Thank you for the comments.

- **Changes in the manuscript:** None

2. Given the age & sex differences present in sarcoidosis and TB patients how were these factors accounted for in the analysis? considering that such factors both have major effects on both immune responses and serum protein levels. At a minimum the comparative analysis between disease and controls could be corrected for age and sex.

- **Answer:** Thank you very much for the feedback. Age and sex were not initially taken into consideration in the analysis and as the reviewer describes, both age and sex can have a significant impact on the circulating proteome. To address this oversight, we performed linear regression for each protein, adjusted for age and sex, as suggested. The differentially expressed proteins (DEPs) identified with the age- and sex-adjusted analyses from both cohorts (sarcoidosis and active pulmonary TB) were again fed into IPA® for pathway analysis. We observed some shifts in the composition of the DEPs, details are described under "Changes in the manuscript". After correcting for age and sex, the BAFF signaling pathway is still significantly activated in sarcoidosis but not in TB. We have now included these additional analyses in the revised version of the manuscript and the data are provided in the newly added supplemental figure 3 in the revised manuscript.

- **Changes in the manuscript:**
  - Line 349 – 356: "Given that age and sex differed significantly between sarcoidosis and active TB patients, an analysis with age and sex adjustment was also performed.

The majority of DEPs in the initial unadjusted analysis for sarcoidosis (191 out of 192 DEPs) and nearly half of those for active TB (42 out of 102) were still observed using linear regression analysis for the protein NPX values. Comparison of analyses between unadjusted and adjusted-derived Ingenuity pathways showed no major differences. Importantly, BAFF signaling and APRIL-mediated signaling pathways were still significantly activated in sarcoidosis but not in active TB (Supplementary Figure 3)."

- We added a new supplementary Figure (Supplementary Figure 3)
- We added Supplementary Table 4 and 5.

3. Also the sample sizes are quite different between sarcoidosis and TB which will have a major impact on the differences observed, the numbers of differences, and strength of differences observed between the two disease groups. One analytical way to address this would be to report effect sizes for the different proteins which is also a better reflection of biological significance than p values. The authors need to acknowledge these limitations in their study design, which means there is likely validity in the commonly observed pathways but interpreting differences is much more challenging.

- **Answer:** Thank you for your feedback and suggestions. Indeed, the sample size is different between the sarcoidosis and TB cohorts. Following the suggestion to down-sample the sarcoidosis cohort to match the TB cohort (see reviewers comment 4 underneath), we have now also added the NPX graphs and corresponding statistical comparisons after random selection of 20 sarcoidosis patients using the DownSample function from the "caret" package in r, presented in the newly added supplementary Figure 7. Additionally, we provide the effect sizes (Cohen's d) of the six proteins belonging to the BAFF signaling pathway as well as several other proteins presented in Figure 5 (IL-16, TRAIL/TNFSF10, CCL11/Eotaxin, FGF2, IL-17C, Fas Ligand, and Granzyme B), both before and after age and sex adjustment and down sampling, in Supplementary Table 4 and Supplementary Table 5. The BAFF signaling associated proteins remain significantly elevated after adjusting for age and sex in a downsampled 20 sarcoidosis patients compared to Dutch healthy controls, with a relatively high Cohens' d values (Supplementary Table 4).

- **Changes in the manuscript:**
  - Line 589 – 599: "It is also important to note that the sample sizes among the groups (sarcoidosis and active pulmonary TB) were unequal. We acknowledge that this is a limitation in the study design. To assess the impact of sample size on our results, we down sampled the sarcoidosis group to 20 randomly selected individuals using the "caret" R package. After down sampling, five out of six proteins belonging to the BAFF signaling pathway remained significantly elevated among sarcoidosis patients (supplementary figure 7 and supplementary table 4). Moreover, using a bead-based immunoassay we demonstrated elevated serum BAFF level in another thirty randomly selected sarcoidosis, which strongly supports the putative biological relevance of the BAFF signalling pathway in sarcoidosis. While suggestive, our findings need validation in subsequent independent larger cohorts, preferably with a balanced study design, to strengthen the robustness of our findings."
  - Supplementary table 4 and 5 were added. These supplementary tables present the fold change as well as corresponding effect sizes (Cohen's d coefficients) for proteins

associated with BAFF signaling, as well as several other proteins specifically mentioned in the results.

4. In line with this could the authors perform down sampling of the sarcoidosis cohort to match the TB cohort size specifically for comparing effect sizes in the B cell signature that they highlight is specific to sarcoidosis, this would give further confidence in the reported results.

- **Answer:** Thank you for the feedback and suggestions. We added further analysis following down sampling of the sarcoidosis patients.

- **Changes in the manuscript:**
  - Line 408 – 417: "To evaluate the influence of the unequal sample sizes on our initial analysis between the sarcoidosis and active pulmonary TB cohorts, we down sampled the sarcoidosis cohort. Using the DownSample function in the "caret" package in R, we randomly selected 20 samples from the sarcoidosis cohort (10 each with and without uveitis). An age- and sex-adjusted analysis of these 20 sarcoidosis patients versus DHC showed that, except for SLAMF7, the other five BAFF signalling associated proteins remained significantly elevated, and subsequently, the BAFF signalling pathway also remained listed as a significantly activated pathway in the IPA® analysis, which again fits the significantly higher BAFF levels we observed in thirty randomly selected sarcoidosis patients (15 with and 15 without uveitis) compared to active pulmonary TB patients and HC (Figure 2B)."
  - Supplementary Figure 7 was added.
  - SLAMF7 was removed when mentioning significant proteins:
    Line 46: "… BAFF, TNFRSF13B/TACI, TRAF2, IKBKG, MAPK9, NFATC1, ~~SLAMF7,~~ and DAPP1"
    Line 472: "..BAFF, TNFRSF13B/TACI, TRAF2, IKBKG, MAPK9, NFATC1, ~~SLAMF7,~~.."
    Line 612-613: "..BAFF, TNFRSF13B/TACI, TRAF2, IKBKG, MAPK9, NFATC1, ~~SLAMF7~~.."

5. Why does the last results section which is a major part of the manuscript only have a supplementary figure, especially when there are only 4 main figures ? However this section is not convincing for the reasons indicated above.

- **Answer:** Thank you for your feedback. We acknowledge that the last part of the results section is important and adds substantial value to the presented analysis. We have now depicted the verification analysis as Figure 5. The selection of these additional proteins is additionally described in the Supplementary Figure 8, but Figure 5 already highlights the essential part of the analysis. We believe this format will enhance the readability of the manuscript.

- **Changes in the manuscript:** None.

Minor:
1. Figures are not numbered in the PDF which is not helpful but I guess this is a journal formatting issue.

- **Answer:** We have uploaded and named the images accordingly as per journal format.

- **Changes in the manuscript:** None.

2. Discussion line 449 typo "the immunostaining staining".

- **Answer:** Thank you for recognizing this. We revised accordingly by deleting the redundant "staining" word.

- **Changes in the manuscript:**
  Line 484: **"**Importantly, *the immunostaining* we conducted on granulomas.."

3. There is no data availability statement in the manuscript which should indicate where the data sets will be deposited to allow other researchers to reuse the data.

- **Answer:** We have included the data statement for the proteomics dataset we have generated and used for the analysis of this study in the methods section.

- **Changes in the manuscript:**
  Line 238 - 239: **"**The proteomics data were deposited into the figshare repository, as part of this record: https://figshare.com/s/c8128b5610fed0023da7."

Reviewer #2 (Remarks to the Author):

1. The results of this manuscript underscore the significant clinical overlap between sarcoidosis and tuberculosis. However, we are reminded that other infections may mimic sarcoidosis, such as syphilis.

- **Answer:** Thank you for the comment. The syphilis test, in the form of VDRL and TPHA, was part of the routine testing we performed on all new uveitis patients, including those who were referred or suspected to have sarcoidosis or TB uveitis. None of the patients tested positive for those tests.

- **Changes in the manuscript:**
  Line 189-191: **"**Complete blood counts, erythrocyte sedimentation rate, syphilis serology (TPHA and VDRL), chest X-ray, and QuantiFERON-TB Gold in-Tube tests were performed for all patients. None of the included patients tested positive for syphilis serology or HIV."

2. To optimally exclude patients with alternative reasons for uveitis, the authors should clearly state how many patients (sarcoidosis, TB) had a "complete" workup with ocular fluid analysis for infectious pathogens and blood tests to screen for TB, syphilis, and autoimmunity.

- **Answer:** Thank you for the feedback. We added this detail in the method section. In all uveitis patients, syphilis and TB test (QuantiFERON-TB Gold in-Tube and chest X-ray) were part of routine uveitis workup, both in the Indonesian and Dutch cohorts. Autoimmunity tests were tailored, however, each of study patients were consulted and assessed by the internist-immunologist or pulmonologist at each study site. Importantly,

patients with coexistence of autoimmune disease (e.g., SLE, rheumatoid arthritis) were excluded from this study. To enhance validity, we carefully selected patients with sarcoidosis and TB based on our inclusion criteria.

- **Changes in the manuscript:**
  Line 187 – 200:
  **“**Patients’ workup.
  All patients underwent a systematic workup as part of the routine diagnostic process at baseline presentation. Complete blood counts, erythrocyte sedimentation rate, syphilis serology (TPHA and VDRL), chest X-ray, and QFT tests were performed for all patients. None of the included patients tested positive for syphilis serology or HIV. Among sarcoidosis uveitis patients, two underwent ocular fluid analysis for PCR testing for herpes simplex virus (HSV), Varicella Zoster Virus (VZV), Epstein-Barr virus, Rubella Virus, and Cytomegalovirus (CMV), along with Goldmann-Witmer coefficient calculation, and neither tested positive for these viruses. Among uveitis patients with active pulmonary TB, five underwent ocular fluid PCR for multiple pathogens (*Mtb*, CMV, VZV, HSV, Rubella, and *Toxoplasma gondii*). One patient tested positive for *Mtb* PCR, and none tested positive for the other pathogens. All patients were consulted to the Department of Internal Medicine (Internist-Immunologist and or pulmonologist) at each study site. None of the included patients had coexisting autoimmune or autoinflammatory diseases upon thorough clinical and laboratory examination.”

3. Otherwise, the study results seem to be consistent with prior reports and identify potential therapeutic targets. As stated by the authors, a larger study will be needed to verify these results.

- **Answer:** Thank you for the feedbacks.

- **Changes in the manuscript:** None

Thank you very much.
Kind regards,

I. Putera, MD
Willem A. Dik, Ph.D
On behalf of all co-authors

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The authors have addressed my concerns.

Reviewer #2 (Remarks to the Author):

The authors have satisfactorily addressed items identified from the original review. No additional concerns.