Author's response to reviews

Title: Posttraumatic stress disorder is associated with an enhanced spontaneous production of pro-inflammatory cytokines by peripheral blood mononuclear cells

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

Thank you very much for your response letter to our manuscript MS: 690147407477006 „Posttraumatic stress disorder is associated with an enhanced production of pro-inflammatory cytokines by peripheral blood mononuclear cells“. We would like to thank the reviewers for their helpful comments and address below the specific points raised by them.

Reviewer #1:

Point 1. As presented in Participants section (page 5), considerable differences exist between groups in gender, age, smoking and drug intake. Authors should put participants’ characteristics in table and compare them between groups with appropriate statistical methods. Additional potential modifiers (confounders) such as alcohol use and physical activity should have been assessed. If not, authors should point it out while discussing limitations of the study.

Response: As suggested, we have added the respective subject characteristics to Table 1 and calculated the appropriate statistics (see Table 1 and page 10ff).

With respect to the potential confounding effects of alcohol abuse and physical activity: Subjects diagnosed with alcohol abuse or dependence were excluded from the study. Unfortunately, we did not assess physical activity and thus are not able to control for a confounding effects of this variable. We have added this as a limitation of our study to the discussion section (see page 15, last paragraph).

Point 2. In Participants section (page 6, first paragraph) authors list exclusion criteria suggesting that they enrolled more participants than reported for the purpose of this study. They even mention 6 participants that were excluded due to the hepatitis. Authors should explicitly state how many subjects were enrolled and how many were excluded due to the exclusion criteria.

Response: As suggested by the reviewer, we explicitly name the number of participants initially enrolled in our study, as well as the number of participants excluded because of the different exclusion criteria (see page 6, paragraph 2):

“Exclusion criteria for the study were intake of glucocorticoids or acute (1 PTSD patient and 2 controls) and chronic (1 PTSD patient and 3 controls) somatic illnesses. In addition, control subjects were excluded...
if they met the criteria for any mental disorder according to DSM-IV (n=4), or reported intake of psychotropic medication (n=2). PTSD patients were excluded if they met the criteria for comorbid alcohol or substance abuse and dependence (n=3) or a current or past history of a psychosis (n=1) according to DSM-IV. Furthermore, participants were screened for possible HIV and hepatitis A, B and C infections. All samples were negative for HIV or hepatitis C. Subjects classified with acute or chronic hepatitis A or B (3 PTSD patients and 3 controls) were excluded from the study, reducing the initially enrolled sample of 44 individuals with PTSD and 39 controls to 35 PTSD patients and 25 control participants.”

Point 3. While listing exclusion criteria authors mentioned “stress-related affective or anxiety disorders”. Neither DSM-IV (which was btw. used for diagnosis), nor ICD-10 clearly defines such disorders (disorders related to trauma/environmental stress are scattered through multiple diagnostic groups). Authors should explicitly list mental disorders that were considered exclusion criteria.

Response: We would like to thank the reviewer for drawing our attention to this point: We have reformulated the respective paragraph according to the suggestion of reviewer 1, by explicitly naming mental disorders we defined as exclusion criteria in PTSD subjects, namely comorbid alcohol or substance abuse and dependence or a current or past history of psychosis according to DSM-IV (see page 6, paragraph 2).

Point 4. Main findings of the study (differences between groups in spontaneous and LPS-induced cytokine production by cultured PBMCs) are based on analyses of a subsample of 16 PTSD patients and 18 control subjects. Authors don’t provide any information regarding demographic and/or clinical characteristics of these specific subsamples and they didn't control for possible influence of these variables in their analyses.

Response: We have added a table with the demographic and clinical characteristic of this subsample (see Table 2). Furthermore, all relevant analyses were calculated as-is and when including sex and smoker/non-smoker status as covariates. Including or excluding these covariates does not influence the main pattern of results; nor are interaction terms between PTSD status and the covariates significant. We have included these calculations to the manuscript and revised our conclusions accordingly (see page 11ff). We would have done the same for age; however, the groups differ significantly on this covariate. In this case, ANCOVA cannot “remove” or “control for” any effect of age: “there is no statistical method that can address the question of whether two groups that differ on variable A would differ on variable B if they did not differ on variable A” [1] quoting [2] -see also other literature referenced by Miller & Chapman [1]. In fact, Miller & Chapman [1] on p. 44 explicitly address the case of differences in age between two groups. This problem is related to Lord’s Paradox [3]. To be on the safe side, we calculated Spearman correlations between age on the one hand and plasma IL-6, IL-8, IL-10, TNF-α and MCP-1 at t1 as well as unstimulated and LPS-induced IL-1β, IL-6 and TNF-α production by PBMCs, in each case separately for PTSD patients and control participants; none of the correlations reached significance (see page 10, first paragraph, page 12, last paragraph and page 15 limitations section).

Point 5. Statistical analyses section (page 8) should be more elaborate. I am not sure which methods authors actually used: parametric or nonparametric. Most of the results that are reported have emphasized F-values (where appropriate) or even t-values (Table 1, supplement 1). This suggests that authors used parametric tests (ANOVA, student t-test). If so, authors should state how they tested for assumptions that should had been met. They should also address the problem of multiple comparisons. On the other hand, authors state that “statistical significance for the immune measures was assessed by nonparametric permutation tests” (page 8). In my opinion, this indeed would be appropriate method for the purposes of this study. However, no p-values derived from permutation tests are emphasized throughout the manuscript.

Response: t-tests were only used in assessing differences in demographic data. All immunological parameters were investigated using nonparametric methods. Group differences were assessed by
generating permutation distributions of the $F$ statistic (which can of course be calculated even if the underlying data do not fulfill the requirements of ANOVA – however, their distribution will not follow an $F$ distribution under the null, which is why we empirically determined the distribution of this statistic). We have elaborated as follows on the permutation approach used: “In each case, the full model and a reduced model omitting the factor(s) of interest were fitted and the statistic of interest (usually an $F$ statistic) was calculated. Next, residuals under the reduced model were randomly permuted 10,000 times. In each case, the randomly permuted residuals were added back to the (non-permuted) fitted values under the reduced model. The resulting randomized dependent values were then again used in fitting full and reduced models, yielding a “permutation” statistic. The $p$ values reported below are given by the position of the original statistic in the empirical distribution of the permutation statistic.” This approach, known as “permutation testing by permuting residuals under the reduced model” is a well-known permutation test procedure [4]. Correlations were assessed using Spearman’s rho (see page 9-10).

In addition, we have changed Table 3 (Table 2 in the original submission) to show medians and quartiles instead of means and standard deviations, in light of the non-normality of the immunological parameters. (We considered changing statistical tests to tests on medians instead of on means, but this appeared infeasible when controlling for covariates.)

As to multiple testing: We now explicitly state which of our results remain significant after correction for multiple comparisons with Holm’s stepwise procedure, applied first for the five cytokines measured in plasma and then for the three cytokines produced by PBMCs measured in presence or absence of LPS (see page 9-12).

Point 6. I don’t think that Figure 1 is appropriate way to present cytokine data. Authors don’t provide any information regarding distribution of their data. Even if we assume that depicted variables are normally distributed, standard errors (whiskers) underestimate dispersion of the data (standard deviation should be used). On the other hand, if nonparametric tests were used (which is not clear), this would imply that data was not normally distributed and in that case other type of diagram should be used (i.e. box and whiskers).

Response: Since data were indeed not normally distributed (as reported above), we have changed the figures, now displaying raw data, jittered horizontally to avoid overlapping points (see Figure 1).

Reviewer #2:

Point 1. The authors set out here to test whether low-grade inflammation, and in vitro production of inflammatory cytokines are activated in a group of PTSD patients compared with controls. This is based on the rationale that previous studies have yielded inconsistent results with regard to plasma inflammatory cytokines. One of the concerns with the current study is that although I agree that more data is needed on peripheral inflammation in PTSD, this study does not really address the problem that is most likely the underlying cause for the current inconsistent literature. The current inconsistency is most likely the result of the fact that the different PTSD populations studied and summarized as having PTSD are too heterogeneous, a problem that is beginning to be resolved with regard to HPA axis activity, but that is not currently addressed in the literature of peripheral inflammation in PTSD. The approach of the current paper is to add MORE data points to our current literature, which is valuable as it will be useful in later systematic reviews or meta-analyses to shift the balance to yes, we have low-grade inflammation vs. no, we don’t, but it remains largely an attempt to replicate earlier studies. A better approach for the future of research on inflammation in PTSD would be to identify reasons for inconsistency, and specifically and strategically target these inconsistencies by comparing different types of traumas, age groups, comorbidities, etc. Nevertheless, this is a valuable addition to our knowledge, just not very novel or innovative.

Response: We feel that our study is more than a mere replication, as it is the first study in PTSD patients
reporting not only LPS-stimulated cytokine production but also spontaneous production of pro-inflammatory cytokines by PBMCs demonstrating an enhanced production of these measures in PTSD patients. Previous in-vitro studies lack such a control condition [e.g. 5, 6] or only report the net effect of LPS stimulation, subtracting spontaneous production from LPS stimulated production before calculating the statistics and thus ignoring the effect of spontaneous cytokine production [e.g. 7]. To give the main finding/contribution of our paper a stronger emphasis we now have slightly modified the title to: „Posttraumatic stress disorder is associated with an enhanced spontaneous production of pro-inflammatory cytokines by peripheral blood mononuclear cells“

However, we agree with reviewer 2 that the inconsistent results with regard to plasma inflammatory cytokines might be due to the heterogeneity of PTSD patients studied such as different type of traumas (e.g. childhood vs. adulthood trauma), time elapsed since the trauma (e.g. populations studied shortly after traumatic experiences or patients with chronic symptoms), comorbidities such as comorbid alcohol abuse/dependence or major depression but also differences with respect to PTSD symptom severity (e.g. studies investigating patients with mild symptoms after single trauma vs. patients with severe trauma and a high symptom score). Moreover, earlier studies investigating plasma cytokine levels consisted mostly of small patient groups, making results prone to the effects of confounding variables (e.g. current infections etc.). Therefore, we believe that a replication of previous research, which our study certainly constitutes insofar as it investigates cytokines and uses PTSD patients as subjects, is certainly justified.

We tried to address these inconsistencies by:

- Examining a thoroughly diagnosed population of 35 severely affected PTSD patients (mean CAPS score = 80) with multiple traumas mainly experienced during late adolescence and adulthood and a chronic disease pattern. As such, our sample explicitly avoids the issue of too much heterogeneity that reviewer #2 very correctly points out.

- Moreover, because of the high variability of cytokine levels, we even repeated the measurement of basal cytokine levels after a one-week interval to test the stability of our measures – an approach not incorporated in previous studies which measured cytokine levels only once.

**Point 2.** Furthermore, it is unfortunate that the in vitro studies were only done on a sub sample of the entire group, further limiting the impact of the results. Related to above comment, it is not really a big step forward to repeat what has been done in previous studies, but to introduce another difference, or more heterogeneity, by using one of many approaches (i.e. PBMC vs. whole blood, incubation times, type and concentration of stimulants). Here again I would argue that yes, this is good additional info, but a study that would bring us forward would identify potential reasons for inconsistency in findings, and derive strategic and targeted tests.

**Response:** Indeed, it is unfortunate that we could characterize cytokine production by PBMCs only in a subsample, which was due to an unfreezing of samples during transportation to the laboratory, which we decided not to further maintain a high quality standard. But again we would like to point out that our approach to test spontaneous production of pro-inflammatory cytokines by PBMCs and the finding of an enhanced spontaneous production of IL-1β, IL-6 and TNF-α is new and not a replication of previous results. Furthermore, we would like to point out that the effects are clearly significant although the N is smaller than in the serum/plasma cytokine investigation which argues for the strength of the effects and is not a weakness per se.

We thank the reviewers for their careful reading of the manuscript and their constructive comments, which we tried to address to the best of our knowledge. We hope that the present version of the manuscript meets the requirements for being published in *BMC Psychiatry*.

Thank you again for your support and advice.
Sincerely,

Iris-Tatjana Kolassa

References: