Author’s response to reviews

Title: Lipopolysaccharide treatment suppresses spontaneously developing ankylosing enthesopathy in B10.BR male mice: the potential role of interleukin-10

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Author's response to reviews: see over
Dear Dr. Takanobu Nakase, Associate Editor,

Please find enclosed the revised version of our manuscript entitled: “Lipopolysaccharide treatment suppresses spontaneously developing ankylosing enthesopathy in B10.BR male mice: the potential role of interleukin-10” which we are submitting for exclusive consideration of publication in BMC musculoskeletal disorders.

The paper clearly demonstrates that repeated systemic administration of microbial component lipopolysaccharide in early adulthood suppresses the development of ankylosing spondylitis in otherwise susceptible B10.BR male mice. In our animal model, the innate immunity and IL-10 cytokine seem to be involved in the disease protective mechanisms. As such this paper should be of interest to a broad readership including those interested in the prevention and therapy of autoimmune disorders affecting musculoskeletal system.

We thank both reviewers for stimulating and encouraging comments and respond to all concerns in a point-by-point manner.

Sincerely,

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Dear Reviewer,

We are grateful for critical reading of our manuscript and we did answer your comments in a point-by-point manner.

Major compulsory revisions:

1. Methods, sections on mice and serum samples: it is unclear which mice were used for the serum studies, because it is unclear how and when their blood was collected. Was the bleeding done at sacrifice, i.e. around the same time as splenectomy? This confusion could be addressed with a figure or table, showing the flow of which mice underwent which intervention at which time.

A new figure (Figure 1) was created which addresses the mouse number issues, time points of LPS administration and serum and spleen collection and also the incidence of ANKENT in a clear and concise way. The sections on mice and serum samples in Methods were completely rewritten. Please see also answer to the comment #10.

2. In Results, paragraph 5 (‘In vitro cytokine response of spleen cells’) the relevant text, including description of Figure 6, is missing. It seems that the text of the previous paragraph was copied.
3. The paper is inconsistent in its handling of the finding that one of the 55 LPS-injected mice developed ANKENT (Results, paragraph 2). The abstract gives the impression that this group did not develop ANKENT at all. Similarly, paragraphs 2 and 4 of the Discussion section include statements that LPS-injected mice did not develop ANKENT. A consistent approach should be followed. Do the authors speculate that the protective effect of LPS had subsided in the mouse, which developed ANKENT at age 9 months? This should be discussed explicitly.

It is true that one of LPS-treated males developed ANKENT at the end of experiment therefore we have replaced the sentence “LPS-treated males remained unaffected by the disease” with “LPS treatment decreased the incidence of ANKENT” in the abstract, the Results section and also modified the above-mentioned statements in the Discussion (paragraphs 2 and 4). Explicitly, the statement “LPS-treated males did not develop ANKENT” was replaced by “the incidence of ANKENT in LPS-treated males was significantly reduced” and “ANKENT did not occur” was replaced by “ANKENT occurrence was suppressed”.

It’s difficult to speculate about the subsiding of the protective effect of LPS as only one ANKENT-positive case appeared at the end of experiment at the age of 9.5 months. However, we consider future experiments which would address this issue by extending the observation period.

4. Currently lacking in the paper is reflection on what it means to compare immunological profiles on a group level, considering the heterogeneity within groups. In other words, how valid is it to consider the immune profile of the PBS-treated group as ‘typical’ for ANKENT susceptibility, if the cumulative incidence of ANKENT only reached 14.2% in this group?

The original goal of the project was to increase the low spontaneous ANKENT incidence and to use the high incidence ANKENT model in further studies. As we did not accomplish this goal the possible future approach could be to differentiate between the ANKENT-positive and the ANKENT-negative mice and treat them separately not as one group. However, we do not know which mice will develop ANKENT thus the only feasible option is to collect sera non-terminally from all mice and match serum samples with ANKENT-positive or ANKENT-negative mice later when the ANKENT occurrence is known and also do full immunological analysis only at the end of experiment.

5. The Discussion section, paragraphs 5 through 12, is difficult to follow. The paper seems to struggle to develop an explanation for how LPS might protect against ANKENT.

The paper discusses in turn each of the group-level immunological differences observed after the 2nd and 4th LPS injections (at about age 4 and 5 months) and also at age 10 months. Sometimes small, inconsistent or even absent effects are given a surprising amount of emphasis. For example, it is unclear why the role of Tregs in allergy is even mentioned. Furthermore, the comparisons of cytokine data from serum and LPS-stimulated spleen are patchy. Also, these two sets of observations are not addressed from a theoretical point of view. What would the authors expect from LPS-stimulated spleen cells from a never, once or twice LPS-challenged mouse at various ages? Were the authors surprised at the discrepancy in IL-10 data between serum and LPS-stimulated
spleen cells? Finally, the discussion of TNF-alpha in paragraphs 9 and 11 seems to be contradictory.

We have rewritten the Discussion section to answer and comply with major comments 5 and 6. We have excluded non-relevant topics and focused on discussing serum and in vitro cytokine data. In addition, we attempt to explain the seemingly contradictory IL-10 data.

6. Due to the unexpected findings (first-ever observation of a protective effect of LPS) and the uncertainties of integrating observations from groups of mice where randomization may have been compromised (see point 9 below), I recommend shortening and restructuring the second half of the discussion section, starting from paragraph 5. The authors might focus first on the immunological effects of LPS treatment as measured on cell subpopulations and cytokine expression, tying together the strongest and most consistent effects and discussing them in relation to the literature. Next, the authors might speculate on how these immunological data might reflect a possible protective effect of LPS on ANKENT susceptibility, keeping in mind the limitations in the current experiments (possible non-comparability of groups, heterogeneity within groups, and the limited number of timepoints when immunological parameters were measured). The current version of the paper mentions many analogies to human disease and animal models; more focus might make the paper stronger. For example the conclusion mentions the hygiene hypothesis but it is not discussed at all.

The Discussion section was rewritten.

Minor essential revisions:

7. In several places the paper refers to numbers of cells, instead of percentages (or proportions), e.g. in the abstract under results. Similarly, in the Discussion, paragraph 6, it is inaccurate to speak of ‘expression of ... cells’.

“Numbers” were replaced by “percentage” or “frequency” throughout the manuscript as we agree with the opponent that the output of flow cytometry analysis should be expressed as a percentage.

8. The mice shown in Figures 1 and 2 should be identified in relation to the experiments in this paper.

The legend of Figure 1 and 2 (now Figure 2 and 3) was supplemented with the identification of the diseased mouse - for the former Figure 1 - “A 7-month-old ANKENT-positive male from the control LPS-untreated group with both paws affected”. For the former Figure 2 - “A control LPS-untreated male with the right paw affected. The left paw is normal.”

9. Since various non-genetic factors have been associated with risk for ANKENT (caging, reference 33; maternal age, see Weinreich et al. 1995; Annals of Rheumatic Diseases 54:754.) it is relevant to describe more details of how mice were divided in groups, i.e. how they were randomized and housed. Were litters split? Were all mice always caged with other males? Was the experiment for observing ANKENT done simultaneously with the experiment, which measured immunological parameters after the second and fourth LPS treatment? Such details will help the reader to form an impression of the validity of comparisons between LPS and control groups as well as the validity of integrating data
from mice observed for ANKENT with data from mice observed only for immune parameters.

All required data concerning non-genetic factors influencing the ANKENT incidence were completed in the Methods, section Mice and also mentioned in the Discussion, paragraph 2. A clear and concise summary of the experiment including timeline, interventions and mouse numbers is provided in a new figure (Figure 1). The authors were well aware of the previously described non-genetic risk factors when designing the experiment and from this point of view the validity of comparison between LPS and control group should not be questionable.

Discretionary revisions:

10 Can the authors clarify why the numbers 55 and 70 were chosen?

Initial numbers of experimental mice were 73 males in the LPS-treated group and 88 males in the control group. As we originally assumed a higher incidence of ANKENT in the LPS-treated group we used slightly lower mouse number (to reach statistical significance). The numbers 55 and 70 mice are the final numbers at the end of experiment from which the ANKENT incidence was calculated. Because the mouse numbers were not well explained and in places were quite confusing we rewrote some paragraphs (Methods-Mice) and included a new figure describing different time points when the mice were sampled.

11. The Methods section, subsection Statistical analysis, first paragraph states that p values ‘within the limits p<0.01-p<0.05 were considered significant’. What is the reason for giving a lower limit? The second paragraph of this section states that standard errors are shown for cell populations. It is more appropriate to show standard deviations. The authors might actually consider showing all the data points, see for example figure 9.7 in D.G. Altman, Practical Statistics for Medical Research, Boca Raton, Chapman and Hall, first edition (1991).

We did not find any reason for giving two p-value limits thus the sentence was changed to “The P values less than 0.05 were considered statistically significant”.

We agree that standard deviations are better option than standard errors. We have modified the graphs and relevant text.

12. It seems that the cytokine production by spleen cells fluctuated a lot, i.e. Figure 6 shows that the average IL-6 values for control mice were about 70, 40 and 200 pg/ml at different time points. Do the authors wish to speculate about the significance of this fluctuation?

The increase of IL-6 level at the end of experiment might be caused by the presence of ANKENT-positive males. We cannot exclude this factor also for LPS-treated group as a single positive case appeared at the end of experiment.

13. This paper is the first report that LPS may protect mice from developing ANKENT, at least for a certain period of time. This was quite unexpected and therefore the authors are not to be blamed for the fact that the immunological observations were not optimally timed to explain such a phenomenon. However, it would be interesting if the authors could briefly propose a direction for future work. For example, do the authors see merit in repeated measurements of serum IL-10 in cohorts of LPS-treated and control mice, during long-term
observation for ANKENT, as IL-6 was measured in reference 11? Do they see value in functional immune assays?

In possible future studies we would like to evaluate the potential positive role of IL-10 using IL-10/-/- or at least anti-IL-10 antibodies. It would be useful to extend observation period as at the end of experiment a positive male appeared in LPS-treated group and the levels of pro-inflammatory cytokines were increasing. Another interesting improvement would be to collect blood non-terminally and match cytokine data with ANKENT-positivity later.

Minor comment:

14. Reference 33 does not present any data about aggression, as the authors imply in the Discussion section, paragraph 3.

We have changed the wording of the paragraph 3 (now paragraph 2) which now explicitly says “the males caged together have a significantly higher risk of ANKENT development than males caged alone” which is in accordance with reference 33.

15. In a few places the English is a little awkward, e.g. in the first sentence of the abstract (‘..ANKENT represents one of animal models...”).

The above mentioned sentence was replaced by “ANKENT is an animal model of...”. Other suboptimal sentences were found and rewritten.

Dear Reviewer,

We sincerely thank for the stimulating and encouraging comments, which helped us to improve the manuscript. Our answers and relevant actions to individual points are stated below.

We agree that IL-10/-/- mouse model would be excellent tool to prove the positive role of IL-10 in the protection of LPS-treated mice against ANKENT development. We plan to go this way in future studies.

Major compulsory revisions for inclusion in the discussion.

It seemed surprising that serum IL-6 in both LPS and untreated mice was elevated at 22-24 weeks.

We might speculate that LPS treatment only delayed the onset of ANKENT as there was an ANKENT case at the end of experiment in LPS-treated group. This would explain increased IL-6 levels in both groups. We included this speculation in the Discussion section.

It would be useful to know if there was a difference in serum IL-6 and TNFa between mice that were affected or unaffected with ANKENT in the control group?
The sera and spleens collected after the second and fourth LPS dose were acquired terminally and before the ANKENT occurred thus we cannot distinguish between ANKENT-positive and -negative males and also the mice analyzed at the end of experiment were evaluated as a whole (as a group). In future experiments we plan to collect blood non-terminally and match ANKENT positivity with serum samples.

The authors state that commensal bacteria play a role in susceptibility to ANKENT. Commensal bacteria may deliver stimuli that will be localized to the gut and the lamina propria whereas systemic injection of LPS will deliver LPS to other lymphoid organs. Could the authors discuss the decision to introduce LPS via this route.

We have previously described that gut microbiota are essential for the development of ANKENT. By systemic administration of LPS, which increases gut permeability (Hietbrink et al. 2009; Mishima et al. 1998; Unno et al. 1997), we planned to enhance the effect of gut microbiota and increase the ANKENT incidence. This is now specifically mentioned in the Discussion and the appropriate references included.

Was there weight loss observed in the LPS administered mice?

We did not weigh mice during the experiment. At the end of experiment there was no significant difference in body weight between groups (5 months after the last LPS injection).

Tolerogenic effects on splenic responses to LPS were not detected by in vitro studies on splenocytes isolated following LPS treatment, were the LPS doses utilized similar to other studies that noted LPS tolerogenic effects?

Mice can be tolerized with an i.p. (or i.v. with a similar effect) LPS administration at the range 1 - 100 ng. Higher doses can induce LPS tolerance but with no extra effect. The protective effect lasts from 60 min to 48 h and is dose-dependent. In our experiments mice were given i.p. 100 ug/20 g LPS. Even such doses induce LPS tolerance however the spleens were always collected 3 days after LPS administration thus the tolerogenic should not be present (M A Freudenberg 1988).


