Author’s response to reviews

Title: Modulation of hepatic PPAR expression during Ft LVS LPS-induced protection from Francisella tularensis LVS infection

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Author’s response to reviews: see over
We have given below the detailed responses in blue text under each of the reviewer’s comments.

Oswald Crasta

Reviewer: Wangxue Chen

Although LVS is highly virulent to mice, particularly by the i.p. route, this strain of the pathogen is highly attenuated in humans and in mice by the subcutaneous route. There is little doubt that i.p. LVS infection in mice constitutes an excellent model for studying the pathogenesis of intracellular bacterial infections, similar to intravenous BCG and Listeria monocytogenes infection models. However, its value as a model for human tularemia remains to be determined.

While it is interesting and potentially significant to identify a significant up-regulation of the fatty acid metabolism pathway genes and the expression of PPARalpha and PARgamma following i.p. LVS infection and LPS pretreatment, the data presented in this manuscript is still preliminary. It is understandable that the authors intentionally avoided presenting any clinical, pathological or bacterial data to reduce the length of their manuscript because these data have been presented in their previous publications. However, it is difficult to get a full appreciation and interpretation of the microarray/qRT-PCR alone without any clinical and bacteriologic data. In addition, the significance of this manuscript is severely diminished by the lack of any functional data to support the potential


We undertook this microarray experiment to identify additional genes whose expression was modified by Ft LPS pretreatment and would alter the course of subsequent infection as the first step in the characterization of novel targets for therapeutic intervention. Although beyond the scope of this study, future experiments are planned that will test the hypothesis that pharmacological intervention with a clinically relevant inducer of PPARgamma (i.e., ciglitazone) will protect mice against an otherwise lethal Ft LVS infection, even in the absence of Ft LPS-pretreatment.
We have added the following text in the manuscript:

**In conclusion:** “Experiments to test the capacity of known PPAR agonists to protect against *Ft* LVS challenge will lead to new therapeutic approaches.”

**At the end of the discussion:** “Future studies aimed at examining the effect of *Ft* LVS or type A strains on the hepatic and pulmonary pathology following cell-specific deletion of PPARα or γ from immune cells are needed to further dissect the role of PPARs in the pathogenesis and prevention of tularemia.”

Minor issues:

Pg 3 (last paragraph): please remove one “i.p.”.

Changed.

Pg 5 (RNA extraction): Was the whole liver or only segments of liver used for RNA extraction? If liver segments were used, please state how sampling in individual mice was standardized.

Whole liver. Made Changes in the section “RNA extraction”

Fig. 4: Please clarify the meanings of the significance symbols. Are they significant from different times post LVS challenge or significantly different between LPS and no-LPS treatment at the same time point.

Clarification is given below. Also made changes in the manuscript in ‘Methods’ (section “Comparison of differential gene expression due to LPS treatment“)

A symbol of asterisk in Figure 4 suggests that the average of the paired-differences (between LPS-treated vs Saline-treated samples at different time points) is significantly different (>1) at p<0.05. A paired t-test is applied to find out: given the data, how likely it is that there is no difference between LPS and no-LPS treatment. Using a p-value threshold of 0.05, we find that it is unlikely for PPARα and γ, i.e., LPS does up-regulate expression of these genes at all three time points.
Reviewer: Anders Sjöstedt

The main problem with the manuscript in its present form is that the key findings presented in figures 4 and 5 are difficult to understand. In figure 4, there is insufficient information regarding the statistical comparisons and it is not obvious from the figure how the comparisons were performed.

Clarification is given below. Also made changes in ‘Methods’ (subsections “Comparison of differential gene expression due to LPS treatment“ and “Comparison of differential gene expression due to LPS treatment and infection on fatty acid metabolism”) and ‘Results’ (section “LPS-induced changes in pathways “)

The comparison for figures 4 and 5 is based on paired t-test, which has been described in greater detail in a new subsections “Comparison of differential gene expression due to LPS treatment“ and “Comparison of differential gene expression due to LPS treatment and infection on fatty acid metabolism” which have been added to the Methods section of the revised manuscript.

Moreover, it is stated that the comparisons were performed using t-test but is this test appropriate for this sort of comparisons?

The basic statistical analysis that was performed on all genes was LIMMA [see references 24, 25 and 26], which is a robust statistical method for identification of differentially expressed genes.

Specific contrasts were conducted on a specific sub set of the genes (e.g., fatty acid metabolism) to address specific questions (e.g., effect of LPS). In such cases, Paired-t-test was applied.

For Figure 4, we asked if LPS pre-treatment was associated with consistent elevation in gene expression at all three time points. For this purpose, a paired t-test was appropriate because it provided a measure of certainty that the gene expression was elevated at all the time points after infection. We discovered that only 2 out of 3 PPAR genes were significantly up-regulated by LPS. This is also consistent with tissue-specific expression of PPAR. For Figure 5, we sought to find the direction of change in gene expression after the mice are challenged with LPS or whole bacterium (i.e., infection). Here, the test was construed as a comparison between two groups (treated and untreated) and paired t-test was considered appropriate.
The information provided in the legend to figure 5 is insufficient since it is unclear which time points that were analyzed and what the data represent.

The two columns refer to two comparisons of treated with control. For the left column, the treatment is 24 hours post-infection while for the right column it is 48 hours post-LPS. For both groups, control refers to no-LPS treated and uninfected mice. Fold change is calculated between treated and control for each gene. We show in this figure that most of the genes participating in fatty acid metabolism are up-regulated by LPS (the right column) but are down-regulated by infection (the left column).

Changes to the legend of Fig. 5 are made.

Also made changes in ‘Methods’ (subsections “Comparison of differential gene expression due to LPS treatment” and “Comparison of differential gene expression due to LPS treatment and infection on fatty acid metabolism”) and ‘Results’ (section “LPS-induced changes in pathways”)

Were PPAR-alpha and -gamma upregulated 0.9 times post-infection and down-regulated 1.2 and 1.1 times post-LPS treatment? It needs to be explained why 0.9 denotes an upregulation.

The numbers indicate relative expression values (ratios of the average intensities) as compared to the control. The numbers >1 indicates up regulation and <1 indicate down regulation.

Moreover, these numbers do not appear to agree with the text in the Results section.

We thank the reviewer for pointing this out.

The numbers in the supplemental table 1, indicate the differential expression values of the pair wise comparisons made between treatments or sample groups (average of three biological replicates). LIMMA was used to test the significance.

In fig 4 and 5, specific contrasts were made where treatments were pooled and tested using paired-t-test in specific comparisons.
We have added more description to remove the confusion as follows:

Made changes in ‘Methods’ (subsections “Comparison of differential gene expression due to LPS treatment” and “Comparison of differential gene expression due to LPS treatment and infection on fatty acid metabolism”) and ‘Results’ (section “LPS-induced changes in pathways “)As described before different types of comparisons were made.