Author's response to reviews

Title: Systemic inflammation links to atherosclerosis via C3aR1; results of a human in vivo LPS infusion study.

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Author's response to reviews: see over
Dear Editor,

Thank you for reconsidering our paper “Systemic inflammation links to atherosclerosis via C3aR1; results of a human in vivo LPS infusion study” (BMC MS: 1079563464463212). Based on the comments of the reviewers we have amended our paper.

Comments made by Dr. Belstrøm.
We would like to thank Dr. Belstrøm for his kind words.

Comments made by Dr. Grond-Ginsbach
The reviewer suggests that we should present the cDNA microarray data from in vivo LPS treated human volunteers. He suggests that we comment on the other genes and work. We should also address the effect monocyte isolation had on the transcription and RNA stability. He also suggests we include our in vitro data. We would like to thank the reviewer for the efforts he has made to improve our paper and the time he spent in doing so. This is our response.

1. We have presented the results of the in vivo experiments in Tables 1 and 2. Because the aim of our paper was to answer the question whether endotoxin exposure in vivo results in changes in monocyte transcriptome that could lead to a more atherogenic phenotype we have chosen to focus our discussion on the role that genes we have identified have on atherosclerosis. However, we have now also added information on the genes for which we did not find a direct relationship to atherosclerosis. Still, because of the possible limitations of our study we have chosen only to discuss the genes which have been differentially expressed both in our in vivo and in vitro work. This is the reason we for instance do not discuss CD14.

2. We believe that the discrepancy in temporal expression of C3aR1 and all other genes except for AKIP is probably due to two facts as stated in the discussion. “This observation is likely due to the sequential activation of pathways after the LPS stimulus. In addition, this might reflect different populations of monocytes contributing to the transcription, since only 45% of monocytes remain in circulation one hour after LPS stimulation”.


3. We would like to point out that no genes were differentially expressed in the control experiments, which indicated that the sample processing had minimal effect on gene expression. We have more clearly incorporated the possible effect of the isolation methods on gene expression and RNA stability in the discussion. Paul Lyons et al. have demonstrated that positive selection by means of antibody-receptor combinations as we have applied does not have adverse influence on cellular transcription and that this is preferred to negative selection in microarray experiments [1].

4. We have incorporated the in vitro study. The methods and results section have been amended. For the methods section we have chosen to create a supplementary section. The results of our in vitro experiments are also available under (ArrayExpress accession number E-TABM-483). We have added the full table in the supplement (table 5). Because we use the in vitro data as an additional filter mechanism to verify our in vivo data, it is beyond the scope and message of this paper to discuss these in detail.

Comments made by Dr. Yang
We would like to thank Dr. Yang for the statistical review and nice comments on the paper.
This is our response.

MAJOR COMPULSORY REVISIONS:
1. A. The first paragraph under data analysis section is a measure of extra quality control for the microarray spots in the data. The software for calculating expression from scanner, GenePix, has its own internal measure of quality control. However, our experience suggested that the measure is not adequate to filter out ('flag') some genes that exhibit poor spot structure. Readings from these spots can produce bias in the analysis as they do not represent the true specific binding. This extra filtering measure that we introduced helps us to identify some more features that exhibit poor structure. In the context of mRNA extracts from blood cells, the extra measures are extremely relevant as some spots in the microarray show poor structure more often than they do for RNA from other cell types [2,3].
B. These criteria were chosen as quality control filtering before statistical analysis is performed. So they do not represent any formal test of differential expression.

C. The linear mixed effects models were considered because we have limited number of samples in our experiments from limited number of individuals. To identify without bias the differentially expressed genes, we need to separate out the 'person' effect from the expression of each gene. The purpose is to identify genes due to the effect of experimental intervention only, and not due to the choice of person we include in the experiment. In the model, the fixed effect is an intercept that represent the differential expression between t=0 and t=1 (or t=0 and t=4), and the random effects are 'person' effects. We consider the 'person' effects as random effects (and not fixed effects) because the individuals are random samples a from population and there is sampling variability associated with the ascertainment.

D. We selected an FDR<0.005, posterior probability of differential expression >0.95, FC is greater then two and present in at least 70% of the arrays. This has been added to the methods section.

2. We have incorporated the in vitro study. The methods and results section have been amended. For the methods section we have chosen to create a supplementary section. The results of our in vitro experiments are also available under (ArrayExpress accession number E-TABM-483). We have added the full table in the supplement (table 5).

3. We agree with the reviewer that the overlap is poor. We have amended the discussion to incorporate this fact and given some reasons why as proposed by the reviewer.

4. Out of the 11 infusion experiments the 5 samples with the highest purity based on FACS and RT-PCR and sufficient yield were chosen for arraying at T=0 vs. T=1 and T=0 vs. T=4 LPS. Similarly 4 out of the 5 were chosen for the control
experiments for the T=0 vs. T=1 and 2 of the 5 samples for the T=0 vs. T=1. RT-PCR was used to confirm the observations from the comparative microarray study in the 6 LPS samples not used for Microarray for the following transcripts at random. No duplication was carried out in the experiments. We have amended the methods and results section accordingly.

5. The genes for RT-PCR confirmation were selected at random. We have added this to the methods section. For VCAN we apologize. In the previous table a synonym CSSPG2 was used, we have altered this to VCAN.

6. We do not think that C3aR1 induces systemic inflammation. What we can see is that it is expressed in monocytes after an inflammatory stimulus, which gives systemic inflammation in humans, based on our clinical results. We only believe that C3aR1 translated the LPS stimulus resulting in systemic inflammation into a possible atherosclerotic mechanism in circulating monocytes. We believe that the discrepancy in temporal expression of \(C3aR1\) and all other genes except for AKIP is probably due to two facts as stated in the discussion. “This observation is likely due to the sequential activation of pathways after the LPS stimulus. In addition, this might reflect different populations of monocytes contributing to the transcription, since only 45% of monocytes remain in circulation one hour after LPS stimulation”.

MINOR REVISIONS:

7. The data uploaded to array express is pending. We will update you as soon as this has been completed.

8. Thank you for pointing this out. The figure was generated from data from the current study. We agree that it was stated in a confusing manner. We have amended the location of the reference.

9. We have amended the figures and now they contain error bars also for the micro array results.
We hope that our response to the reviewers is satisfactory and that you will consider our work for publication.

Kind regards,

On behalf of all authors,

Suthesh Sivapalaratnam

References:

