Author's response to reviews

Title: Induction of the interleukin 6/ signal transducer and activator of transcription pathway in the lungs of mice sub-chronically exposed to mainstream tobacco smoke.

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Version: 3 Date: 27 April 2009

Author's response to reviews: see over
RE: MS: 1446438040232357 - Induction of the interleukin 6/ signal transducer and activator of transcription pathway in the lungs of mice sub-chronically exposed to mainstream tobacco smoke.

Dear Dr. Talbot,

We are very happy to hear that our manuscript may be acceptable for publication in BMC Medical Genomics. We thank you for the very helpful comments from the Editors and reviewers. We have carefully examined the reviewers’ comments and made changes to our manuscript accordingly. Please find below our detailed response. We thank you for your time and consideration and we hope that our manuscript is now acceptable for publication in BMC medical Genomics.

Best regards,
Sabina Halappanavar

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Reviewer-1
Major comments:
1) The background section provides only a superficial generalized introduction and does an inadequate job of reviewing current knowledge regarding gene expression changes that occur in lung tissues following exposures to cigarette smoke. If the focus of the study is ultimately on the response of IL-6 and the JAK/STAT pathway, then a review of what is known of those interactions in the context of cigarette smoke exposures should be presented. Given the extensive literature regarding both response to cigarette smoke as well as molecular genetic changes that occur in airways during smoking-associated disease development, it is not informative to simply state that cataloguing which genes respond to cigarette smoke will identify those key to disease development.

Agreed.
The suggested background information on IL-6 and JAK/STAT3 pathway has been added to Discussion section instead of background section as specified.
Page 20, second paragraph. Page 19, last part of the first paragraph.
Page 4 and 5.

2. There is insufficient detail regarding the selection of the mouse model, the exposure protocol and selection of dose. Does this mouse model develop smoking-associated lung tumors following exposure? Was the exposure a whole body exposure or nose-only? It is widely known that whole body exposures result in considerable oral carcinogen uptake through grooming. An acknowledgment of the limitations of mouse models of human lung cancer should be included (citing, for example, Witschi, 2007 “Tobacco smoke-induced lung cancer in animals--a challenge to toxicology (?)” Int J Toxicol. (2007) Jul-Aug;26(4):339-44). How was the relevant dose determined? Two cigarettes per day for a whole-body exposure represents a very low level of exposure. Was the actual amount of exposure estimated, for example, via serum cotinine levels? COHb levels? Has this level of exposure been shown to evoke a toxicologically meaningful (toxic rather than adaptive) response?

Agreed.
We have modified the methods section to indicate that the selection of dose and time points was based on previously published results where serum cotinine levels were measured and were found to be consistent with that seen in regular active human smokers.
Mouse strain selected – did not want to use an entirely inbred mouse (wanted a broader measure of response) and wanted to include C57BL because probes are based on this strain’s published genome.
Page 7, second paragraph.

3) Aside from its similarity to earlier studies, the primary weakness of the current study is the use of whole lung samples for microarray analysis, which raises significant issues for interpretation of the microarray data. Lung is composed of many widely differing cell types, each of which would be expected to display a different spectrum of expressed genes at baseline. Furthermore, it would be expected that different lung tissues would experience a different level and possibly type of exposure, and thus respond via transcription of different and/or different levels of genes. Following tissue homogenization, all such differences are lost. While it may be argued that matched controls may account for this cellular heterogeneity, it is likely, for example, that airway epithelial cells respond significantly to the exposure, but their signal may be obscured by the diluting effect of mRNA from cells that are not exposed. (see discussion of this issue in Fielden and Zacharewski, 2001: “Challenges and limitations of gene expression profiling in mechanistic and predictive toxicology,” Toxicol Sci. (2001) Mar;60(1):6-10.) Furthermore, changes in cell populations can likewise obscure transcriptional responses (see below). These issues are of increased importance when changes in mRNA levels are modest, as in the current study.

Agreed.
We have discussed the issue in Discussion section briefly.
Page 23, second paragraph.

4) The discussion of the statistical methods should be simplified and made much more clear. For example, the rationale for use of “date of hybridization” as a statistical parameter should be at least briefly discussed.

Agreed.
We have added more details in the method section.
Page 9, second paragraph.

5) Table 1 presents “79 genes” that were differentially expressed with statistical significance in at least one treatment. The changes in many if not most of these genes are extremely modest. The analysis of BAL fluid indicated that the number of inflammatory cells were elevated 1.5-fold after 6 weeks, and as much as 3-fold after 12 weeks exposure. The shift in cell populations following exposure would seem to be able to account for slight changes in “gene expression” without invoking any transcriptional responses to cigarette smoke. This criticism is particularly relevant since the authors chose to place considerable emphasis and devote a considerable part of the manuscript to a discussion of the small changes they detected in IL-6 and JAK/STAT signaling (which is likely from the
monocyte/macrophage population) and the apparently paradoxical finding of SOCS3 mRNA upregulation. The complexity and cell-type specific responses that are characteristic of this pathway would seem to be difficult to analyze in an experiment that utilizes whole lung as starting tissue. (see O’Shea and Murray, 2008: “Cytokine signaling modules in inflammatory responses” Immunity. (2008) Apr;28(4):477-87.)

Agreed. However, there was no change detected in IL-6 levels in BALF. Due to the limited amount of BALF we could not repeat these experiments to get statistical significance. As a result we have not reported these results.

6) Figure 6: No information is provided on how the levels of proteins detected by Western blotting were quantified. Considering the importance attached to the reported changes in the level of SOCS3 protein, a gel photo should be included to reinforce the extremely modest differences among the SOCS3 levels displayed for each of the 4 experimental conditions. Error bars are included in this figure. Was a protein sample analyzed for each individual mouse?
   Agreed
   We have changed the Methods section and added more details of how the protein levels were quantified. Also, a gel photo has been added to Figure 6.
   Protein samples were analysed for each individual mouse. This info has been added to the methods section.
   Page 11, second and third paragraph.

7) The authors devote the bulk of the discussion to gene expression changes relating to IL-6 and SOCS3. IL-6 is a well-accepted systemic marker of inflammation and has been reported elevated in the serum of smokers in multiple studies. The authors provide a detailed discussion of aspects of current understanding of the interaction between IL-6 and SOCS3 (and other downstream effectors), interactions that may indeed regulate the long-term nature of an inflammatory response to environmental agents such as cigarette smoke. They concluded that due to the lack of an observed increase in pulmonary neutrophils after 6 or 12 weeks exposure, and a modest decrease in the level of SOCS3 protein, that there is likely inhibition of pulmonary inflammation under these low exposure conditions. Inhibitory effects of cigarette smoke on immune functions are well documented, as is the somewhat paradoxical observation of chronic systemic inflammation among smokers. The authors should include a discussion of this literature and compare with their results.
   Agreed
   A paragraph is added to the Discussion section.
   Page 21, second paragraph.

Minor comments
1) Figure 1: Panel A is not informative as there is no annotation. What is the meaning of the colors? Panel B would be much more useful if the actual treatments were identified rather than simply numbers. Also the gene descriptions appear to be raw output from the clustering program. Gene names along with a functional category would greatly improve this figure. Again, there is no description as to the meaning of the colors.

**Agreed**

*We have changed the Figure-1 to incorporate the suggested changes.*

2) Figure 4: looks like the colors indicating the different treatments are reversed.

**Do not agree**

*We have checked and the colours indicating different treatments are not reversed.*

**Discretionary changes:**

1) Figure 2: Provides a nice integration of the categories and interactions of differentially expressed genes detected by microarray analysis. It would be even more helpful to provide the 6-wk and 12-wk results of the expression data on this figure. For example, next to the SOCS3 icon “(+1.41 / +1.70).”

**Do not agree**

*We think the figure will become too busy. Besides, Table-1 provides details of fold changes on all genes.*
Reviewer-2

Major Comments
1. This deals with the design of the study. Gene expression is measured in total lung at 3 h after the last MTS exposure, and 6 and 12 weeks thereafter. Exposure to MTS affects the cellular composition of the lung (as is also shown by the authors in Table 4), and merely this change may have its effect on the gene expression profile. F.i. only after 12 weeks exposure an increase of mononuclear cells was observed, and also the IL-6/SOCS3 effects were only present at that moment. The effects on inflammatory pathway may thus well represent the influx of mononuclear cells. The cross-contamination of lung with blood cells could have been reduced when the gene expression was conducted on the lungs after alveolar lavage, as that removes most of the infiltrated cells. Why was that not done? All these aspects must be described in the discussion.

Agreed
We have added a paragraph in the Discussion section.
Page 23, second paragraph.

2. In figure 1, the numbering of the columns cannot be correct! 7-8 are in a separate branch of the tree, not 1-2, as is written by the authors on page 12

Agreed
We have changed the figure accordingly.

Minor:
3. No histopathology was conducted on the lungs. This weakens the study, as it could further substantiate the findings.

Agreed
Histopathology was conducted on lungs derived from mice exposed to 8 weeks of smoke similarly by the same exposure system has been published by our collaborators previously. They have shown no overt pathology in the lungs of smoke exposed mice. Therefore, we have not conducted histopathology.

4. Page 16 line 7, mentions that early upregulation of genes was found. Upregulation after 6 weeks of exposure cannot be called early, as that should be within hours or maybe a day, and requires more time-dependent analyses.

Agreed

5. The effect on gene expression at 3 h after the last exposure, may very well reflect the last exposure (thus is a measure of an acute effect) and not an accumulative effect of the 6 or 12 weeks exposure. The authors should discuss this.

Do not agree
If the effects seen were a mere reflection of the last exposure, we should have seen identical profile of gene expression in 6 and 12 weeks. But that is not the case here. Also, Acute exposure to cigarette smoke in rat lungs are shown to suppress IL-6 activity in lungs or induce IL-6 degradation in BALF. Here we report upregulation of IL-6 and its activity.

o What is the relevance for humans, especially with respect to the observation that at 6 weeks after exposure all effects on gene expression are gone? This should be discussed.

Agreed
A paragraph has been added to the Discussion section
Reviewer 3
No changes suggested
Reviewer 4

Major comments
1. The authors have identified a set of genes that are differentially expressed after 6 or 12 weeks of tobacco smoke exposure, with return to baseline post-cessation. Comparison of these gene expression changes to previously published microarray datasets in human airway or lung tissue exposed to cigarette smoke would demonstrate the potential clinical relevance of their findings. While the previously published in vivo human datasets represent chronic exposure and often involve a single cell type (e.g. airway epithelium), demonstrating overlap in gene expression changes would strengthen the physiological relevance of this smoking mouse model as it is unclear if the degree of exposure mimics that seen in human smokers (i.e. no carboxyhemoglobin measurements reported). Additionally, there are several microarray studies of human lung epithelial cell lines exposed acutely to smoke in vitro that could be used, although the physiological relevance of these datasets is less clear. The authors could use an approach like Gene Set Enrichment Analysis (GSEA) in order to provide statistical confidence for the enrichment of their gene set (79 genes) within the previously published studies.

Agreed
We have added a paragraph in the discussion section comparing our data with already published gene expression data sets in the literature. Page 24.
We have added few lines in Methods section to indicate that the doses chosen were based on the previously published work from our collaborators, where cotinine levels were measured and were found consistent with the levels seen in regular active smokers. Page 7.

2. The microarray studies were performed on whole lung tissue and it is unclear whether changes in gene expression with smoke exposure are due changing cell types (i.e. inflammatory cell infiltrates) within lung. The authors have shown a change in mononuclear cell counts (but not neutrophil counts) on BAL, but were there inflammatory cell changes seen histologically in the lung tissue post-exposure? The authors did validate select changes at the protein level by western, but no immunohistochemistry was performed to identify the cell type responsible for the changes. A description of the histological changes to the lung post-exposure and cessation from exposure would help strengthen the biological conclusions of this manuscript including their statement in the discussion that there is “an absence in inflammation in mouse lungs following 12 weeks of exposure to MTS” (p.20).

Agreed
A paragraph has been added to the discussion section discussing the limitation of the study. Page 23.
We have not shown neutrophil counts as the results were totally nil.
Histopathology was conducted on lungs derived from mice exposed to 8 weeks of smoke similarly by the same exposure system has been published by our collaborators previously. They have shown no overt pathology in the lungs of smoke exposed mice. Therefore, we have not conducted histopathology.

3. The authors report that samples group by their respective treatment groups when clustered across either all genes on the array or the 79 differentially expressed genes (p.12), but only show results for the 79 genes in Figure 1. The authors should also show, via PCA or via clustering dendogram, the more dramatic finding that samples cluster by treatment group across all genes on the array. Additionally, it is difficult to follow how samples cluster within Figure 1A and whether the mice studied 6 weeks post-cessation cluster with controls. The color scheme in their dendogram is difficult to follow as no color legend is provided.

Agreed
We have changed the figure 1 to incorporate the suggested changes. We have also changed results section to highlight the changes.

Minor issues:

2. Were there any quality control metrics employed to filter out poor quality microarrays?
Agreed
More details on statistical methods used to filter out poor quality microarrays has been added to the Methods section. Page 9.

3. Figure 4 should include the results for the BAL neutrophil counts as the authors use this negative finding to support their conclusion regarding the absence of inflammation in lung.
Do not agree
We have not shown neutrophil counts as the results were totally nil.