Reviewer's report

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.

Version: 2 Date: 16 December 2008

Reviewer: Gary M Hellmann

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General Comments:

Halappanavar et al. have employed gene expression profiling using Agilent oligonucleotide microarrays to investigate responses to sub-chronic cigarette smoke exposures in mouse lung, as well as recovery following a period of cessation. Exposures to the smoke from 2 cigarettes per day for 6 or 12 weeks resulted in 79 genes being differentially regulated. The authors focused on genes related to the IL-6/JAK/STAT signaling pathway for confirmation by RT-PCR and additional protein analysis by ELISA and Western blot. The authors concluded that their data indicates a lack of inflammation from the tested exposures, and propose that a decrease in proteins such as SOCS3 may protect against an inflammatory response at subchronic doses of cigarette smoke. While the goal is to provide insight into lung injury and inflammation induced by mainstream tobacco smoke, this study does not add significantly to the body of literature that currently exists using similar techniques. In addition, methodological shortcomings, an inadequate review and summary focusing on relevant background literature, and an emphasis on select expression changes that are modest and sometimes equivocal at best significantly diminish enthusiasm for this manuscript.

Gene expression profiling of lung tissues exposed to cigarette smoke has been conducted in numerous studies using both experimental animals as well as human tissues. For example, Stevenson and co-workers (“Comprehensive gene expression profiling of rat lung reveals distinct acute and chronic responses to cigarette smoke inhalation,” Am J Physiol Lung Cell Mol Physiol. (2007) Nov;293(5):L1183-93) identified time-dependent differentially regulated genes involved in stress response and inflammation. These changes were correlated with histopathological alterations and changes in cytokine response. Meng and co-workers conducted a 3-week nose-only comparative inhalation study of cigarette smoke and LPS in mice, analyzing whole lung samples by Affymetrix GeneChip microarrays. While LPS stimulated a vigorous inflammatory response, the number of inflammation-related genes upregulated in response to cigarette smoke was small. Cell counts in BAL also showed a marked decrease in the number of neutrophils in this group compared with an LPS exposure. However, pulmonary macrophages were increased as was the level of IL-6. The authors speculated that smoke-exposure suppression of acute immune and inflammatory
responses may represent a mechanism for COPD development in smokers
(Meng et al. “Gene expression profiling in lung tissues from mice exposed to
cigarette smoke, lipopolysaccharide, or smoke plus lipopolysaccharide by
(“Modification of gene expression of the small airway epithelium in response to
analysis of small airway epithelial cells in phenotypically normal human smokers,
finding differential regulation of genes involved in inflammation, response to
xenobiotics, etc. These and other previously published studies are similar, if not
more comprehensive in scope, design, and results.

It is recommended that the authors consider the following issues when
re-evaluating their study and manuscript:

Major compulsory revisions:

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.

Methods:

1) 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
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?

2) 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
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.

3) 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.

Results:

1) 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,
Apr;28(4):477-87.)

2) 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?

Discussion:

1) 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.

Minor Essential Revisions:

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.

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

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests: I serve as a consultant on legal matters that include smoking and health.