Author’s response to reviews

Title: The rs361525 polymorphism does not increase production of Tumour Necrosis Factor Alpha by monocytes from Alpha-1 Antitrypsin Deficient subjects with Chronic Obstructive Pulmonary Disease

Authors:
Jennie Gane (jenniegane@doctors.org.uk)
Robert Stockley (rob.stockley@uhb.nhs.uk)
Elizabeth Sapey (e.sapey@bham.ac.uk)

Version: 1 Date: 12 Oct 2015

Author’s response to reviews:

10th October 2015

Dear Dr Kim,

Reference: JNRB-D-15-00013

Thank you for your letter dated 23rd September, regarding our submitted manuscript:

“The rs361525 polymorphism does not increase production of Tumour Necrosis Factor Alpha by monocytes from Alpha-1 Antitrypsin Deficient subjects with Chronic Obstructive Pulmonary Disease- a pilot study.”

Jennie Gane, MBChB; Robert A Stockley, DSc; Elizabeth Sapey, BSc, MBBS, PhD

We wish to resubmit a revised manuscript together with a response to the reviewers’ comments. We have outlined responses to the reviewers’ comments below in a point by point manner.

Reviewer One

Strengths of the manuscript are that there is a sound rationale for conducting the study as: 1) prior studies show that COPD patients with AATD and chronic bronchitis having this SNP have 100-fold more TNF-alpha in their sputum than subjects without this SNP; and 2) TNF-alpha likely contributes to the inflammatory process in the lungs of patients with AATD and COPD. The studies are novel, the methods are sound, and the experiments have been conducted on primary cells from patients that have been carefully phenotyped and well matched. Also, the
results are clear cut, and the manuscript is clearly written, and the limitations of the study have been discussed. Overall, this is an important negative finding that should be in the public domain.

Major comments: None

Response: We thank the reviewer for their kind comments.

Minor discretionary revision: 1. Among subjects with the TNF-alpha polymorphism, AATD patients having the chronic bronchitis phenotype are the subgroup that have 100-fold higher sputum levels of TNF-alpha. Although it is recognized that AATD is relatively uncommon and only a small number of subjects could be studied, Table 1 shows that ~50% of the AATD patients with the polymorphism have chronic bronchitis. Can the authors run a subgroup analysis to see whether monocytes from the 4 chronic bronchitis patients with the polymorphism have higher (or trends towards higher) TNF-alpha expression or secretion than cells from the 9 AATD patients without the polymorphism or the 5 chronic bronchitis group without the polymorphism?

Response

We have run a subgroup analysis as described above. When considering comparisons of the concentration of TNF-α protein, there was no trend observed or statistically significant greater TNF-α production by the monocytes from patients with the polymorphism and chronic bronchitis compared to those without the polymorphism or those with the polymorphism but no chronic bronchitis phenotype. When considering TNF-α mRNA expression, again no statistically significant differences were found. In four of the eleven tested time-points/conditions there was a trend for slightly greater TNF-α mRNA expression in the monocytes with the polymorphism and chronic bronchitis phenotype compared to both other groups. However as the differences were slight and not consistent, there were only 4 in the polymorphism/chronic bronchitis group and the subject groups no longer matched, we feel that no reliable conclusions can be drawn regarding the hypothesis that it is only monocytes with the rs361525 polymorphism from patients with chronic bronchitis that express more TNF-α and hence we have not included this subgroup analysis in the manuscript. We have made a comment in the manuscript as to why this could not be conducted with any scientific rigour.

Reviewer Two

1. I appreciate the difficulties in obtaining and culturing primary human bronchial epithelial cells, but given the previous observation that subjects with the rs361525 SNP have significantly increased TNF-α levels in sputum, while systemic levels are not elevated, the manuscript would be stronger if it incorporated also studies on at least sputum-isolated macrophages.
Response:

We agree with Dr Plate’s comments that it would be interesting and important to study cells isolated from the airway, for example, sputum macrophages, given the lack of difference in TNF-α output observed in blood-derived cells. However, the initial aims of the current experiments were to study the effects of increased TNF-α output in the rs361525 polymorphism monocytes (had this been shown) on monocyte and monocyte-derived macrophage functions relevant to COPD, for example, phagocytosis and pro-inflammatory cytokine production. For this reason, induced sputum analysis was not part of the study protocol. Future experiments should concentrate on airway derived cells, both immune and structural, but will take considerable time to conduct in full. We believe it is important in the meantime for our current findings to be published as part of a brief report as this will not detract from future work and will add to the body of evidence studying the effect of this polymorphism in blood monocytes.

2. The negative result might be due to a statistical type II error, given the small size of the cohort and the high inter- and intra-subject variability. I would therefore suggest that this study be treated as a small pilot study, indicating this in the title and in the text.

Response

We agree with the reviewer’s comments and have used the term “pilot study” in the title, abstract and body of the text. The possibility of the study being underpowered, for example as a result of intra-subject variability in TNF-α production, is addressed in the discussion.

3. It might be useful to add an in silico study of the effect of the minor allele of the SNP on transcription binding sites and a brief discussion of this in the context of monocytes and macrophages and other relevant cell types.

Response

In-silico analysis of the effect of the A-allele on transcription factor binding sites has recently been conducted and published by other authors (Kiss-Toth et al, reference 14 in the manuscript). They also found that LPS led to greater expression of the TNF-A gene using a reporter gene assay in murine macrophages with the A allele. Possible explanations for why we did not observe higher TNF-α production after LPS exposure in human monocytes with the polymorphism are discussed in our manuscript and highlight the importance of investigating the effect of this and other SNPs using a variety of methods. As such we believe that this brief report as it stands, contributes to knowledge in this field and highlights some of the inherent challenges in investigating the effect of SNPs on cytokine function, in specific cells and disease states.

4. I recommend using at least 2 validated stably expressed reference genes. This will provide statistically more significant results and help detecting small expression differences.

Response
Whilst we appreciate that it would be desirable to normalize each gene of interest against multiple reference genes, this was not practical in the presented studies as limited RNA was available (all PCR reactions were conducted in singleplex reactions to avoid the problems associated with multiplexing—e.g. DNA polymerase saturation, primer interactions etc, and to use multiple reference genes would have consumed a lot more of each RNA sample). However, experts have stated that where an individual reference gene can be shown to be stably expressed in the model under study then it is acceptable to use only one normaliser, particularly when any slight genuine variability in reference gene expression is outweighed by much greater changes in the gene of interest between samples. For example, a 10 fold variability in expression of the reference gene is less important if changes in the gene of interest are 100 fold (Huggett et al, reference 11). Dedha et al compared the expression of a panel of commonly used reference genes in whole blood and peripheral blood mononuclear cells under a variety of conditions and subject characteristics. They classified stable reference genes as those in which the average fold change of that gene from the mean expression was less than 2 and the maximum variability in fold change less than 5 (reference 12). In our experiments GAPDH was found to be stably expressed in a random selection of 64 samples (from different subjects and under a variety of experimental conditions) with a mean fold change from the mean CT value of 1.7 and a maximum fold change of 4.6. As GAPDH was stably expressed in these validation experiments we opted to use just one normalizing gene.

5. The rs361525 SNP is commonly indicated in the literature as being at position -238 while the authors indicate it as at position -237. Please clarify.

Response

Both -237 and -238 are quoted in the literature and depend on whether the first nucleotide in the promoter region of the TNF-A gene is labelled as 0 or -1. We have changed the text to -238 in the manuscript as the studies we have quoted in the paper also refer to it as -238.

We hope that the responses outlined above answer your reviewers comments but would be happy to provide more information if needed. Thank you for considering our responses to the reviewers’ comments and our revised manuscript. We look forward to hearing from you in time.

Yours sincerely,

Dr Elizabeth Sapey