**Author's response to reviews**

**Title:** The pathogen recognition sensor, NOD2, is variably expressed in patients with pulmonary tuberculosis.

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**Author's response to reviews:** see over
The pathogen recognition sensor, NOD2, is variably expressed in patients with pulmonary tuberculosis. Sanjay Lala, Keertan Dheda, Jung-Su Chang, Jim F Huggett, Louise U Kim, Margaret A Johnson, Graham AW Rook, Satish Keshav, Alimuddin Zumla

Responses to reviewers

Thank you for reviewing our paper. We hope that these responses adequately address the reviewers concerns, which has provided helpful insights into improving the paper.

Reviewer 1: Bernhard Ryffel

Comment 1: The BAL samples from controls and TB patients differed significantly in absolute numbers of leukocytes, lymphocytes and macrophages, but the exact number of the latter is not given, no mention of the amount of epithelial cells is made, at least the counts have to be given.

Our response: We quantitated the total leucocyte count by flow cytometry using anti-CD45 antibody, and lymphocyte counts by using anti-CD3, anti-CD4 and anti-CD8 antibodies. We did not directly count neutrophils and monocytes/macrophages, but the proportion of these cells can be inferred by subtracting the total lymphocyte count from the total leucocyte count (BAL consists of ~ 95% macrophages, ~ 5% lymphocytes, 1% neutrophils, 0.1% eosinophils and very scanty epithelial cells; in fact from our ongoing work epithelial cells constitute a fraction of 1% of total cells). However, NOD2 mRNA is most prominently expressed in monocytes and there is a much lower level of expression in lymphocytes and virtually none in neutrophils (Ogura 2001), and it is therefore very likely that monocytes and alveolar macrophages account for the majority of NOD2 mRNA expression.

Nevertheless, we did not count respiratory epithelial cells in the lavage specimens, and we agree that this data would have assisted with the interpretation of the data. NOD2 mRNA is upregulated in immortalised bronchial epithelial cells infected with Streptococcus pneumoniae (Opitz 2004) and it would have been very interesting to determine whether MTB regulates NOD2 mRNA expression in respiratory epithelial cells: this information would have been especially useful for those patients with very high levels of NOD2 mRNA expression. We have added an explanatory paragraph in the ‘Discussion’ section.

Comment 2: Only transcriptional data are given for TLRs, it would be interesting to see whether membrane expression is altered.

Our response: We agree. However, we recovered a limited number of cells from BAL fluid and PBMCs from patients with PTB, and analysed transcriptional responses only. We have used this same cohort for other studies (published as Dheda 2005, Chang 2006) and thus we had limited biological material to work with. Obtaining further lung samples was logistically impossible.
Comment 3: Functional tests would be very useful: In vitro restimulation of blood leukocytes with TB antigens to test the production of proinflammatory cytokines in the patient population.

Our response: We recovered limited numbers of cells from patients and could not perform many functional assays. In our experience, revival of stored leucocytes obtained from BAL fluid for use in functional assays has proved very difficult.

Comment 4: In the paragraph “Signalling pathways” it is unclear what was the sample size, the stimulant (MDP?), and no results are shown. Further, high level of FLICE are reported, but no data are given.

Our response: We compared NOD2 mRNA expression with the transcriptional responses of selected cytokines, toll-like receptors and molecules involved with apoptosis in BAL-derived leucocytes obtained from 15 patients with pulmonary TB and 6 healthy controls. No functional tests were performed on these cells (see above). In BAL-derived leucocytes obtained from PTB-affected patients, we could not demonstrate any significant correlation between NOD2 mRNA expression and:

1. TLR6 expression (p=0.16);
2. TLR7 (p=0.13);
3. TLR1 (p=0.31) or its splice variant (previously described in Chang 2006) TLR1s (p=0.14);
4. IL-4 (p= 0.90);
5. IFN(γ (p=0.56);
6. TNFα (p=0.60);
7. TNFR1 (p=0.44);
8. TNFR2 (p=0.77);
9. Cas 8 (p=0.56);
10. FLICE (p=0.47);
11. Bcl-2 (p=0.63);
12. Bax (p=0.98);
13. Bfl-1 (p=0.30).

Similarly, in BAL-derived leucocytes obtained from healthy volunteers, we could not correlate NOD2 mRNA expression with transcriptional responses of the molecules listed above. Two patients, who had high levels of NOD2 mRNA expression in BAL-derived leucocytes, also had high levels of FLICE expression (See Figure below). However, NOD2 mRNA levels did not correlate with FLICE transcriptional responses and we did not include this figure in the manuscript.
Comment 5: Pathogen-induced NOD2 transcripts were investigated in control blood leukocyte, no data are given.

Our response: We have included this data in the manuscript (Figure 5). We analysed NOD2 transcriptional responses in healthy donor-derived PBMC that were stimulated with suspension of living mycobacteria (MTB H37Rv or M vaccae). In general, mycobacteria induced modest, but non-significant increases in NOD2 mRNA expression. In one healthy donor (Donor 1), mycobacteria induced marked increases in NOD2 mRNA expression. Thus, it appears that, in a small minority of patients, mycobacterial antigens induce an increase in NOD2 mRNA expression for reasons that are, as yet, unknown.

Comment 6: Last, figure 2 and 3 are inversed and one part is missing.

Our response: We have corrected this.

Reviewer 2: Giovanni Ferrara

Comment 1: data on NOD2 expression in BAL cells could be biased by the different cellular populations in TB patients and control. Is it possible to present the data correcting the results for the differential cell counts? (e.g. for alveolar macrophages?)

Our response: Please see our response to a similar concern raised by Reviewer 1.

Comment 2: the number of patients and controls enrolled should be reported among the results.

Our response: Addressed in manuscript.

Comment 3: It would be correct to describe the methods of in vitro experiment of infection with mycobacteria, in particular the number of PBMCs infected in every well and the multiplicity of infection used. Given the correlation between NOD2 and TLR2 and TLR4 in alveolar cells, I wonder if the Authors performed some experiments stimulating PBMCs with LPS and LAM, even if not considered strictly necessary for this study.
Our response: We have added these methods to the manuscript. We did not stimulate PBMC with LPS or LAM.

We trust that the reviewers will be satisfied with our reply and we will be happy to provide further assistance if needed.