Author's response to reviews

Title: Induction of beta Defensin 2 by NTHi requires TLR2 Mediated MyD88 and IRAK-TRAF6-p38MAPK Signaling Pathway in Human Middle Ear Epithelial Cells

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Version: 2 Date: 2 April 2008

Author's response to reviews: see over
April 1, 2008

BMC Infectious Diseases
Editorial Office

Re:

Dear Sir/Ma’dam,

I would like to submit the revised manuscript to be considered for publication in the journal of BMC Infectious Diseases. We have revised the manuscript to reflect all critiques and suggestions made by the reviewers.

I do hope that you will find the revised manuscript acceptable for publication. Should you have any questions, please do not hesitate to contact me using the information provided below.

Sincerely,

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Reviewer's report (#1)
Title: Induction of beta Defensin 2 by NTHi requires TLR2 Mediated MyD88 and IRAK-TRAF6-p38MAPK Signaling Pathway in Human Middle Ear Epithelial Cells
Version: 1 Date: 26 January 2008
Reviewer: Oren Froy
Reviewer's report:
The authors nicely demonstrate the signaling pathway leading to hBD-2 induction in response to WCL of NTHi. The experiments were performed carefully and the results are convincing. However, the manuscript, in my opinion, should be revised to have a better flow.

Major Compulsory Revisions
1) The Introduction does not cover the field that is presented in the results. The Introduction should deal more with the MyD88------p38 pathway. This pathway is well characterized and should be mentioned in the Introduction, so that when the reader sees those molecules in the Results section he/she will have an idea about the logic behind the experiments. For examples the authors describe this pathway briefly in the dominant negative plasmids section. This description should be referenced and transferred to the Introduction.
Response ➔ We agree with the reviewer and have reflected this in the introduction.

Discretionary Revisions
1). Figures should be rearranged: Fig. 6B should be combined with Fig. 3 to show the importance of TLR2; Fig. 4A and B should be combined; Fig. 4C can be omitted; Fig. 5 A and B should be combined and moved to Fig. 4; Fig. 6 A should be part of Fig. 4. I think this rearrangement would give the paper a better flow. Of course, the corresponding parts in the text and the references to Figures should all be changed accordingly.
Response ➔ We agree with the reviewer and have rearranged and consolidated the figures according to the reviewer’s suggestion.

Minor Essential Revisions
1) There are more than three defensins isolated at the peptide level (Yanagi et al., Respir Res, 2005 and references describing epididymal beta-defensins) this should be corrected in the Introduction.
Response ➔ We added the following statement in the background section “To date, multiple β-defensin genes from epithelial and epididymal cells have been identified [23-25]. Among them, four of epithelial β-defensins (HBD 1-4) have been characterized at the peptide level [23, 24, 26].”

2) Lipooligosaccharide (LOS) should be spelled out in the text.
Response ➔ As suggested, LOS has been spelled out in the first appearance.
Reviewer's report (#2)
Title: Induction of beta Defensin 2 by NTHi requires TLR2 Mediated MyD88 and IRAK-TRAF6-p38MAPK Signaling Pathway in Human Middle Ear Epithelial Cells
Version: 1 Date: 12 February 2008
Reviewer: pearay L logra
Reviewer's report:
This is an interesting study in which the investigators have demonstrated Induction of b-defensin 2 via TLR 2 sactivation following in vitro and in vivo exposure to NTHi.

Specific comments:
1. Background Para 2, line 1. Palate, Lung and nasal epithelium are not simply "molecules". This sentence could be rewritten.
Response → We have rewritten the sentence as follows: “Innate immune molecules such as lysozyme, lactoferrin, PLUNC (palate, lung and nasal epithelium clone) and defensins are produced by the mucosal epithelial cells and provide the host with continuous innate immunity against a variety of invading pathogens [14, 15].”
PLUNC is a relatively new innate immune molecule composed with 256 amino acids and has molecular weight 26713 Da. This macromolecule is expressed in the upper airways and nasopharyngeal regions and has innate immune function.

2. Materials and methods . Para 1, Line 11. Did you examine the cellular debris also for b defensin 2 induction. para2 ,line 2. MEEC cell line is an immortalised line with human papilomavirus. It would be important to to know if primary cell lines behave simmilarly and if viral induced cell activation is necessary for the TLR activation in this setting. It would also be helpful to know if the b defensin induction is LPS dependant.
Response → We collected the cellular debris, called Pellet (P), and tested it along with other preparations in Fig 1A, as a function of induction level of beta-defensin 2 by NTHi 12 LOS. We also tested the effect of LPS on the induction of beta-defensin 2 in HMEEC (data was not presented in this paper) but the result was similar to that of LOS treatment. Although we agree with the reviewer with the fact that testing and confirming the observed results in primary cell is very important, we cannot comply due to the difficulty in obtaining healthy human middle ear cells because of the restriction of human subject regulations by IRB. To verify our observation that beta-defensin 2 induction by NTHi in HMEEC is a common phenomenon in airway system, we used human lung carcinoma cell-line, A549 as our in vitro reference and C57BL/6J mouse as the in vivo model (Fig2 A, B, and C).

3. Figure 4. The abbreviations used here should be explained somewhere in the text or in the figure title.
Response → The full names of signal transduction proteins were explained in the results section, however, we also added the full names in Fig 4 for consistency.
IRAK1: interleukin-1 receptor associated kinase 1
TRAF6: tumor necrosis factor receptor associated factor 6
MKK3/6: MAP kinase kinase 3/6

4. Figure 7. Although the data are reasonably straight forward it would be helpful to provide a unifying concept as to how all the intermediate pathways of signaling cascades are orchestrated in the eventual b defensin 2 activation. Is it possible that the
The principal mechanism is signal transduction for example at the level of Nf-kB. The authors should discuss the possibility and revise the discussion.

Response → Fig. 7 has been removed as suggested.

5. The study has demonstrated b defensin 2 mRNA activation. Does such increased message in fact result in increased b defensin 2 soluble product.
Response → Yes, NTHi12 treatment increased beta-defensin 2 protein production (using an ELISA assay) in HMEEC compared to non-treated HMEEC. The results are presented in Fig 4E.

6. It is not clear what particular component of the NTHi is responsible for the defensin activation. Is bacterial replication in the cells required for such activation.
Response → We believe bacterial replication in the cells is not required for the beta defensin 2 induction by NTHi since the bacterial whole cell lysate induces beta defensin 2. However, finding and identifying the responsible ligand for beta-defensin 2 induction is beyond the scope of our present study.

7. The manuscript is little too long with many figures. The manuscript could be shortened and the discussion limited to data reported here. Figure 7 could be deleted, unless it provides a more data based unifying summary of b defensin activation.
Response → We have consolidated some figures and removed Fig. 7 as the reviewer recommended.

Reviewer's report (#3)
Title: Induction of beta Defensin 2 by NTHi requires TLR2 Mediated MyD88 and IRAK-TRAF6-p38MAPK Signaling Pathway in Human Middle Ear Epithelial Cells
Version: 1 Date: 19 February 2008
Reviewer: Joseph E Kerschner
Reviewer's report:

Minor Revisions
The authors present an elegant study examining the induction of beta-Defensin 2 (HBD-2) and signaling pathways associated with this. There are a couple of comments which should be addressed as the manuscript is prepared for publication.

There are a few grammatical and typographical errors.
Response → The manuscript has been revised to reflect the corrected changes.

The authors could provide the reader with some brief information on the current state of research into soluble macromolecules from NTHi in the Discussion section as they propose (and demonstrate with data) that this is the likely candidate from NTHi as the initiator of HBD-2 up-regulation.
Response → We have added several NTHi macromolecules identified for vaccine candidates in the discussion. Those macromolecules include adhesins, high molecular
weight adhesins, pilus proteins, outer membrane proteins and LOS. To find the beta-defensin inducing ligand is our next aim and will report later.

The authors demonstrate that the 3 strains that they employed were similar in up-regulation of HBD-2 but there should be some comment as to why these strains were selected and if there are possibilities that other strains would behave differently.

Response The three strains were selected because they are the most commonly used laboratory NTHi strains. Although there are many other NTHi strains associated with OM, and those strains may behave differently, testing these strains are beyond the scope of the current study.

The authors should comment on the dose selected (5 µg/ml) of NTHi whole cell lysate (WCL).

Response The NTHi WCL dose was selected according to the results obtained from our preliminary experiments. The dose provided an optimum induction of HBD-2 and/or IL-8 without cytotoxic effect against HMEEC.

The authors should comment on the antibody concentrations (isogenic, TLR2 and TLR4) selected in the antibody blocking experiments and whether this could have impacted the results in Figure 3.

Response The antibody concentrations used were the recommended amounts by the manufacturer, eBioscience. Our preliminary experiments with 10 µg/ml, and 20 µg/ml of antibody showed no significant difference between the two treatments. We therefore used 10 µg/ml as the concentration of choice.