Author's response to reviews

Title: Intracellular expression of toll-like receptor 4 in neuroblastoma cells and their unresponsiveness to lipopolysaccharide

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Author's response to reviews: see over
Dear The Editor,

Please find the revised manuscript “Intracellular expression of toll-like receptor 4 in neuroblastoma cells and their unresponsiveness to lipopolysaccharide” written by Ferdaus Hassan et al. We agreed with both of the reviewer’s comment and according to their suggestion we have changed our manuscripts and major changes made in the text are underlined.

I hope that our revised manuscript is suitable for BMC Cancer. Thank you for handling our manuscript.

Yours sincerely,

Re: BMC Cancer

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Response to reviewer #1 Peter Tobias

Major Compulsory Revisions:

Minor Essential Revisions:

Response: Thank you very much for the critical comments and thoughtful suggestions. We have revised the manuscript according to your comments and responded to them. The major changes made to the text are underlined. The discussion on the unresponsiveness of NB-1 cells to LPS has been added in the “Discussion”.

Response to reviewer #2 Holger Heine

Major Compulsory Revisions:

Thank you very much for the critical comments and thoughtful suggestions. We have revised the manuscript according to your comments and responded to them. The major changes made to the text are underlined.

Fig-2: -as control that the anti-TLR4 antibody actually detects cell surface TLR4, U937 cells should be used as positive control.

Response: Human peripheral blood mononuclear cells were used as positive control for TLR4 expression. Anti-TLR4 antibody positively stained peripheral blood mononuclear cells but not NB-1 cells. According to the reviewer’s comment we modified the text with regard to positive control.

-since mRNA expression does not necessarily correlate with protein expression at least the expression of CD14 can be easily investigated in the same experiment.

Response:

NB-1 cells were positively stained by anti-CD14 antibody and the data was added to the text.

Fig. 3: Negative control (i.e. lanes with everything but cDNA) should be included in the figure.

Response: We agree with the reviewer's comment. However, our experimental procedure is one step RT-PCR. Therefore, we included one lane without reverse transcriptase enzyme (i.e. only RNA) instead of cDNA and changed the figure accordingly.
Figure 4 and 5:
It is clearly seen that the LPS preparation used in this experiment activated the cells in terms of I-κB degradation. Is the activation stronger when higher LPS concentration (i.e. 1 µg/ml) are used? Since these cells may not express cell surface CD14, the sensitivity towards LPS could be greatly reduced as compared to monocytes. In addition, it is well known that these commercial LPS preparations not only contain LPS but also other TLR ligands such as lipopeptides. Thus, in order to exclude that this weak activation is induced by other ligands, purified LPS preparation should be used.

**Response:** When LPS at 5 µg/ml was used, such high dose of LPS failed to activate NF-κB in NB-1 cell line. The unresponsiveness of NB-1 cells to LPS is not dependent on the LPS concentrations used. With regard to commercially available LPS preparation, the reviewer indicated the possibility that contaminants might induce partial activation of IκB. We think that it is unlikely because 50-fold high concentration (5 µg/ml) of LPS preparation did not activate it and other LPS from *E. coli* O111 also showed the partial activation. Further, we had performed the silver staining of our LPS preparation and it showed a beautiful ladder pattern of LPS. However, the possibility that contaminants might induce partial activation of IκB may not be excluded. The possibility has been stated in the text of “Results”.

-the luciferase assay should serve as indication that NF-κB is not activated by LPS in these cells, However, the sensitivity of these particular assay appear to be very weak. Even the positive IL-1β is only able to induce about 3.5 fold or 2 fold activation of the NF-κB reporter, respectively. In the other papers, activation is the range of 10-100 fold is not unusual. Thus, the overall sensitivity may be to low to detect LPS-induced activation.

**Response:** Sensitivity of reporter gene assay greatly depends on transfection efficiency of cell types used. Transfection efficiency might be quite high in some cell types but not in others. In neuroblastoma cell SK-N-MC, only 2.5 fold induction of NF-κB activity is observed on the average. Therefore, neuroblastoma cells might have quite low transfection efficiency and hence low activation of NF-κB in the reporter gene assay.

**Fig7:**
-given the fact that NB-1 cells express only intracellular TLR4, activation of MAP kinase may take considerable longer than in U937 cells. Therefore, a kinetic up to 2 hours should be performed. Does activation of ERK1/2 not occur as well?

**Response:** We agree with the reviewer and extended the time up to 2 hours as the reviewer suggested. We showed the results in text.

**Discussion:**
-the clear lack of IRF-3 might be responsible for the inability to activate the MyD88-independent pathway. However, since MyD88 is expressed at least mRNA level, this should be sufficient to induce cytokine production. In order to really exclude activation of
these cells by LPS, the lack of cytokine induction (i.e., IL-8) should be demonstrated as well.

**Response:** We agreed with the reviewer and checked the production of TNF-α in response to LPS. The results have been added in the “Results”.

**Minor Essential Revisions:**

Page 2, Lane 22-23: line changed to “Collectively, NB-1 cells are capable to avoid their response LPS” as suggested.

Page 4, Lane 115: Changed this line to “Confirm the lack of TLR-4 expression in neuroblastoma cells” as suggested.

Page 8, 119: Commonly? We mean, both the cell line.

Page 8, 120: We changed the subtitle to “Intracellular but not cell surface expression of TLR4 in NB-1 cells” as suggested.

P9, 121: We changed this line to “…that mRNAs of the three molecules are expressed in NB-1 cells” as suggested.

P11, 16: We changed this line to “that LPS induces phosphorylation of IRF-3 in U937 but not in NB-1 cells” as suggested.

P11, 17: Sorry for our careless mistake. We changed the word to IRF-3.