Author's response to reviews

Title: Poly I:C enhances cycloheximide-induced apoptosis of tumor cells through TLR3 pathway

Authors:

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

The second revised manuscript entitled “poly I:C enhances cycloheximide-induced apoptosis of tumor cells through TLR3 pathway” is submitted by Qun Jiang and her colleagues for your consideration for publication in BMC Cancer.

In the second revised manuscript, the authors provided the data as supplementary material, indicating that poly I:C and CHX had no effect on TLR3 expression in the Hela cells and MCA38 cells.

**“Additional file 1 –**
Supplementary figure 1: Analysis of polyI:C and CHX effects on TLR3 expression in Hela cells and MCA38 cells. (A) mRNA levels of TLR3 in Hela cells and MCA38 cells treated with poly I:C (100 μg/ml) for 72 hours were detected by RT-PCR. (B) FACS analysis of intracellular TLR3 in Hela cells and MCA38 cells treated with CHX (2.5 μg/ml) for 24 hours was shown.”

Besides, point-by-point response to the criticisms from referees has been addressed as follows:

Q1. The authors use flow cytometry to assess TLR3 protein expression. >From the methods section, it appears that they used live cells. The cellular location of TLR3 can be either cell-surface or intracellular. Intracellular TLR3 would not be detected using flow cytometry on live cells. Does the level of TLR3 expression differ if cells are permablized, indicating the presence of intracellular TLR3?

Answer 1: We showed the results of intracellular staining of TLR3 in tumor cell lines in Fig 1 B. The level of intracellular TLR3 expression in certain cell lines is higher than the level on the cell surface.

Since we used poly I:C/CHX as extracellular stimulation, it is presumed that surface-located TLR3 functioned as the main receptor recognize poly I:C in our study.

Referee’s concern:
In a generally accepted concept, polyI:C is internalized by endocytosis and recognized by endosomal TLR3. Therefore, this assumption is misleading. The surface and intracellular expression of TLR3 should be indicated.

**Answer:** We had made revision in the 3rd paragraph of discussion. (listed in the table)

Q2. The investigators incubate tumor cells with PIC for 72 hours and then access cell viability. PIC induces TLR3 expression in some cells. Does the level of TLR3 expression change in response to PIC incubation in the tumor cell lines?

A2: We used RT-PCR to detect mRNA level of TLR3, and didn’t find notable changes in tumor cells stimulated with poly I:C or CHX or both for 72 hours. We will provide the data in the supplementary figure if it is required.

**Referee’s concern:**
The authors should at least state this fact in the text.

**Answer:** We have stated this in the result part 2 (listed in the table), and the data was provided in the Additional Figure 1A.

Q4. Does the addition of CHX block TLR3 expression or PIC-induced increases in TLR3 expression?

A4: The result of FACS analysis showed that the addition of CHX didn’t block TLR3 expression on the surface of Hela cells and MCA38 cells. Besides, we didn’t find notable changes of TLR3 expression in tumor cells stimulated with poly I:C, CHX, or both. We will provide the data in the supplementary figure if it is required.

**Referee’s concern:**
The data indicating that CHX did not block surface and intracellular TLR3 expression should be provided.

**Answer:** We have stated this in the result part 2. (listed in the table), and the data was provided in the Additional Figure 1B.

<table>
<thead>
<tr>
<th>1st revised manuscript</th>
<th>2nd Revised manuscript</th>
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<tbody>
<tr>
<td><strong>Results</strong></td>
<td><strong>Poly I:C treatment caused tumor cells more sensitive to CHX-induced cell death</strong></td>
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tumor cells (Hela cells and MCA38 cells) became dramatically lower in the presence of poly I:C (Fig2). We also compared the expression level of TLR3 in poly I:C- or CHX- treated Hela cells and MCA38 cells with PBS-treated control cells, but found no remarkable changes (see Additional file 1).

<table>
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<th>Discussion</th>
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<td><strong>The 3rd paragraph of discussion</strong></td>
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<td>Besides TLR3, PKR, the cytoplasmic helicase family proteins (retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5)) also serve as dsRNA pattern-recognition receptors [1, 25], which are reported to trigger different signaling pathways from TLR3 [26]. Although dsRNA has been shown to induce apoptosis apparently through multiple pathways such as PKR- or caspase-dependent apoptosis in several cell types [3, 4], RIG-I/MDA5 is reported to recognize dsRNA in the cytoplasm, and TLR3 resides both in intracytoplasmic and on the cell surface of the tumor cells as shown in our study, which implicates that the extracellular stimulation with poly I:C/CHX induces tumor cells apoptosis mainly via TLR3 pathway. Moreover, results of our present study on the apoptosis in tumor cells transfected with vector expressing poly I:C show that intracellular dsRNA induced tumor cells apoptosis mainly via RIG-I/MDA5 pathway.</td>
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Thank you.

Sincerely,

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