Author's response to reviews

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

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

A 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*.

Toll-like receptor 3 (TLR3) plays an important role in innate immune responses against dsRNA viruses, which was considered to be mainly expressed in immune cells and some endothelial cells. This study investigated the expression and proapoptotic activity of TLR3 in human and murine tumor cell lines. It’s shown that TLR3 are widely expressed on human and murine tumor cell lines, and activation of TLR3 signaling in cancerous cells by poly I:C made Hela cells (human cervical cancer) and MCA38 cells (murine colon cancer) become dose-dependently sensitive to protein synthesis inhibitor cycloheximide (CHX)-induced apoptosis. Blockade of TLR3 recognition with anti-TLR3 antibody greatly attenuated the apoptosis-improving effects of poly I:C on tumor cells. Moreover, IFN-β production was induced after poly I:C/CHX treatment and neutralization of IFN-β slightly reduced poly I:C/CHX -induced apoptosis. This study demonstrated the proapoptotic activity of TLR3 expressed by various tumor cells, which may uncover a new range of clinical applications for TLR3 agonists as an adjuvant of certain cancer chemotherapy.

In the revised manuscript, the authors supplement results of analysis of intracellular TLR3 expression in tumor cell lines (Page 7, the 3rd paragraph and Fig.1 B), induction of IFN-β in tumor cells stimulated by poly I:C/CHX ( Page 8, the last paragraph and Fig 5 A ) and IFN-β
neutralization assay (Page 9, the first paragraph Fig 5 B). Thus the discussion part was revised accordingly.

The authors provided a point-by-point response to the concerns of 3 referees as follows:

**1. Response to Compulsory Revisions proposed by Reviewer Kiyoshi Takeda (Referee 1):**

1. Unlike the previous studies (Ref 12, 13), TLR3 stimulation itself does not induce apoptosis. The authors should make this point clear.
   **R:** We pointed out in the result part (Page 7, the last paragraph and Page 8, lane 5 to 10 ) that poly I:C alone could not markedly induce apoptosis of tumor cells. In the revised manuscript, we also put enough emphasis on this point in the abstract (Page 2, lane 19) and discussion part (Page 9, lane 16 to 19).

2. It is very ambiguous why the authors use CHX, but not other apoptosis-inducing agents, to induce apoptosis. The authors should state the reason why CHX is used.
   **R:** We didn’t mean to study the role of TLR3 pathway in certain cancer chemotherapy at the beginning. Some results of our study on the cross-talking between TLR3 signaling and TNF-α signaling pathway in certain tumor cell lines showed that the apoptosis of Hela cells could be directly induced by poly I:C/CHX with or without TNF-α. Since the findings might be important to those with closely related research interests, we took the following study, using CHX.

3. It has been shown in other studies (Ref 12, 13) that TLR3 directly triggers apoptosis, and that TLR3-dependent induction of type I IFNs is responsible for apoptosis induction. CHX is predicted to inhibit de novo protein synthesis of type I IFNs, resulting in decreased sensitivity to apoptosis. But this is not the case. As the authors describe in the Discussion, they should show that CHX unexpectedly increases protein expression of IFN-alpha/beta by ELISA.
   **R:** It’s difficult to detect the production of type I IFNs in tumor cells by ELISA, but we did find the induction of IFN-β by poly I:C/CHX through western blot assay (Fig 5 A). We conjectured that it could be explained as super induction, which has already been described in the Discussion.

4. I wonder how apoptotic cells are counted in Fig. 3 B, C. Are these Annexin V-positive cells? If so, data in Fig. 3B should fit with those in Fig. 3A.
   **R:** We treated all cells except for Annexin V and PI-double negative ones as apoptotic cells, so we counted apoptotic cells as follows:
   \[
   \% \text{ (apoptotic cells)} = 1 - \% \text{ (Annexin V/ PI double negative cells)}
   \]

5. The authors analyze surface expression of TLR3 in Fig.1. However, TLR3 is known to be expressed in the endosomal compartment. Therefore, intracellular staining of TLR3 should be performed.
   **R:** 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.
6. In page 3, lane 2, ‘dsRNA virus’ should be ‘viral dsRNA’.
R: It has been corrected in the revised manuscript. (Page 3, lane 2)

7. On page 8, the authors describe that RIG-I also initiates cellular responses. However, a recent study (Nature 441, 101, 2006) showed that another RNA helicase MDA-5 recognizes poly I:C.
R: It has been corrected in the revised manuscript. (Page 10, 2nd paragraph)

2. Response to Compulsory Revisions proposed by Reviewer Massimo Tommasino (Referee 2):

(A) To increase the weight of the study it is suggested to check the effects of poly I:C and CHX on the other cancer cell lines.
R: We described the effects of poly I:C and CHX on the other cancer cell lines, both in the result and discussion part (Page 8, lane 10 to 12 and Page 10, the 3rd paragraph). We didn’t carry out more study on these cell lines because we didn’t find notable apoptosis related to TLR3 pathway in these cell lines. We will provide the data in the supplementary figure if it is required.

(B) The authors should also perform additional experiments using anti a/b interferon receptor to evaluate the possible involvement of the interferon pathways in TLR3-induced apoptosis.
R: We took the additional experiments suggested and results were showed in Fig 5 B.

3. Response to Compulsory Revisions proposed by Reviewer Margaret Offermann (Referee 3):

1. 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 permeabilized, indicating the presence of intracellular TLR3?
R: 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.

2. 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?
R: 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.

3. The authors access the effect of PIC on CHX-induced cell viability. Seventy-two hours is a long
exposure to CHX, and the dose of CHX used is low. How much protein synthesis is inhibited at this dose?

R: We didn’t expect that CHX inhibited most protein synthesis in the response of the cells to poly I:C, for the purpose of studying the role of TLR3 pathway in tumor cells apoptosis. We also tried to avoid the apoptosis of tumor cells attributed to the problem of synthesis of protein required in maintaining cell viability. So the dose of CHX we used (far less than 10 μ g/ml ) was not high enough to put much effect on protein synthesis.

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

5. The authors report that antibodies to TLR3 block the ability of PIC to enhance CHX-induced cell death. Are the antibodies affecting known TLR3 signaling pathways such as the activation of NF-kB or IRF3?
R: We didn’t have enough time to make signaling events clear at present (some colleagues in our lab is going deep), but we believed that antibodies to TLR3 probably would affect TLR3 signaling events induced by poly I:C through blockade of the receptor. For instance, the finding of the production of IFN- β induced by poly I:C/CHX indirectly implicated the activation of IRF3, which probably could be prevented by blockade of TLR3 with the antibodies.

All authors who have actively participated in this study agree with the submission of the manuscript to *BMC Cancer*. The work has not been published elsewhere, either completely, in part, or in another form and that the manuscript has not been submitted to another journal and will not be published elsewhere within one year after publication in this journal.

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Thank you.

Sincerely,
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