Author's response to reviews

Title: Immunotherapy using slow-cycling tumor cells prolonged overall survival of tumor-bearing mice

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Author's response to reviews: see over
Cover Letter

I ensure that we have adhered to ARRIVE and PRISMA guidelines. The manuscript has been modified by the 'Edanz' editor according to the suggestion of the reviewer. We would like to thank reviewers for the positive and constructive comments. A point-by-point response to the concerns was listed as follows.

Response to the comment of Reviewer XiubaoRen:

Comment: In this paper, the authors proposed an interesting hypothesis that vaccination with the slow-cycling CT-26 tumor cells could generate effective anti-tumor response both in vivo and vitro which is somewhat related to up-regulated expression of MHC-II and co-stimulatory molecules on surface after chemotherapy. Their findings are novel and of high clinical significance. The experimental was well-designed and the data were well explained. The English writing is acceptable. But there is a minor revision for this paper: In order to indicate the relationship between chemotherapy and increased proportion of slow-cycling subset in total tumor cells, the authors should explain if the absolute number of slow-cycling cells increased, or more fast-cycling cells converted to slow-cycling cells after chemotherapy. Anyway, the paper is of considerable importance to be published in BMC Medicine.

Response: First of all, we would like to thank the helpful suggestion of Professor Xiubao Ren. In the drug treatment assay, the proportion of slow-cycling subset increased significantly both in vivo and in vitro. And we assume that the proportion increases was caused by the apoptosis of fast-cycling tumor cells which are sensitive to cell cycle-dependent chemotherapeutics (5-Fu). Drug treatment caused the apoptosis of most fast-cycling cells, thus induced the reduction of the whole cell number (As we can see in Figure 3B and Figure 4B). However, slow-cycling cells (insensitive to 5-Fu) survived from drug-treatment. Thus, we can conclude that although the absolute number of slow-cycling cells was unchanged, but their proportion increased because of the reduction of total cell number.
Response to the comment of Reviewer Yukai He:

Comment: It is known that, after selection by many rounds of chemo or radiation therapy and after going through the process of immunoediting in tumor bearing host where the tumor cells have been sculptured by host immune system, re-emerging tumor cells will be different from original tumor cells. In this manuscript, the authors tested a novel idea that slow-cycling tumor cells may provide more relevant Ag to protect tumor relapse and generate more clinically relevant responses to control tumor growth. The idea is excellent and deserves a careful and well designed investigation, which could generate significant impact on tumor immunotherapy. Thus, the reviewer believes that this is a very interesting study that will provide values to this field. However, several issues can be clarified to strengthen the manuscript. In addition, this manuscript requires some professional editing in order to be published in this Journal.

Major issues:

Comment 1: Will the slow-dividing tumor cells change their character with time? In another word, if you keep culturing the slow-cycling cells, will they become fast-dividing or vice versa?

Response: Thanks very much for the good question. To answer this question, an added experiment had been done. Drug-treated tumor cells which were demonstrated to be slow-cycling (high DiI-retaining cells) were kept culturing for 7 days, and Di content was then assessed by flow cytometry. The results showed that after continuous culture, Di+ cell frequency in drug-treated tumor cells reduced obviously. We assumed that a part of slow-cycling tumor cells would differentiate into fast-cycling cells. However, there were another population of them would renew themselves and keep the character of stem cells. Results are shown in supplement Fig 1 and Fig 2.
Supplementary Figure 1 - Results of flow cytometry immediately after drug treatment.

Supplementary Figure 2 - Results of flow cytometry after 7 days culture.

**Comment 2:** The viability of each population should be examined. The Dil high population may be more viable. Staining of apoptosis related protein may be helpful.

**Response:** That is a helpful suggestion, and we agree with the reviewer that staining of apoptosis related protein may be helpful to examine the viability of the population. However, in previous study, the viability of the two populations also had been assessed by Trypan Blue Dye staining before inoculation. The viability of each population was more than 90%. Moreover, MTT assay had been done to determine the proliferation activity of the two populations although the results were not added into the paper. Both of them showed high viability 4 days after cell sorting.
(supplement Fig 3). And DiI- tumor cells showed a shorter period of cell dividing. Thus, we consider that these data could indicate the viability of the two populations although we would apply staining of apoptosis related protein to our future studies.

**Supplementary Figure 3** - Proliferation assay of DiI+ and DiI- cells.

**Comment 3:** The high tumor intake after inoculation of the Dil+ cells indicates that they maybe better escape the immune system, which contradict the findings that drug treated CT26 tumor cells (which is also slow dividing) have high MHC II and CD86 (which means they should be more immunogenic).

**Response:** Thanks for the professional comment. We accept the opinion that the high tumor intake after inoculation of the DiI+ cells indicates that a better escape from immune system. However, we don’t think this contradict our findings. And I would like to explain this from 2 aspects.

(1) Although DiI+ cells have a little higher MHC II and CD86 compared with DiI- cells, the expression level was much lower than antigen presenting cells (APCs), which means the weak increased expression may not improving immune response in naïve mice. And viable tumor cells can induce a strong immunosuppression of host through various approaches, such as secreting immunosuppressive cytokines, inducing the generation of suppressive immunocyte, immune tolerance and apoptosis of effective T cells. As we all know, CTL kills tumor cells by recognizing MHC I - antigen peptide complex. However, our data showed that
MHC I expression on the surface of DiI+ tumor cells was decreased (Table 3), which means a less sensitivity to killing by CTLs. This data indicated that DiI+ cells may escape the immune system through many ways. Thus, we assumed that the immune response DiI+ cells induced would not be strong enough to overcome the immunosuppression, which means a better escape from immunosurveillance.

(2) When DiI+ cells were inactivated artificially and reintroduced into a tumor bearing mice as vaccine with adjuvant, their capability of inducing immune response would improve significantly. That was for several reasons: first of all, inactivated tumor cells would induce much lower immunosuppression compared with viable tumor cells; secondly, repeated vaccination might enhance the stimulation of effect T cells, and slight up-regulation of MHC II and CD86 might be helpful to induce anti-tumor immunity in repeated vaccination, although the impact may be very weak. More importantly, gene chip assay has been performed to investigate the difference of mRNA expression between DiI+ and DiI- cells. And some tumor associate antigens (TAA) were found to be much higher expressed in DiI+ cells than in DiI- cells. When we immunized mice with inactivated DiI+ cells, these high expressed TAAs were enriched and presented to the immune system. Simultaneously, antigens equally expressed by both DiI+ and DiI- cells were also exposed to the immune system. Thus, vaccinations using inactivated DiI+ cells would induce immune response targeting both DiI+ and DiI- tumor cells, which indicates a better therapeutic effect.

Comment 4: The authors did not touch the issue of Ag repertoire change between slow dividing and fast dividing cells. But could that be one of the reason why drug treated tumor cells be more immunogenic? It is understandable that extensive array assay may be needed to identify the difference of Ag repertoire. Thus, some explanation in the discussion should be added to address this question.

Response: Thanks for the valuable suggestion, and an explanation about Ag repertoire change has been added to the discussion according to this comment of Professor Yukai He. Actually, gene chip assay has been performed to investigate the
difference of mRNA expression between slow dividing and fast dividing cells. Now we have found a series of over expressed genes in slow dividing cells, and the related proteins of some genes had been reported to be tumor associate antigens. However, further research is still in progress.

Comment 5: The data showed that the drug treated CT26 cells have higher MHC II and CD86 (table 3). The authors believe that this will attribute to the induction of higher immune responses. If so, this may be significantly different from what has been observed in vivo. The relapsed tumors after chemo or radiation therapy often escape the immune system, indicating that in vivo cancer chemo treatment rather select the tumor variant that may be invisible to the immune system. The authors should provide some explanation.

Response: Thanks for the professional comment. We would like to explain this from 3 points:

(1) As I mentioned in response to comment 3, the slight up-regulation of MHC II and CD86 may not impact the immune response in non-human tampering condition. We assumed that the immune response drug-treated cells induced would not be strong enough to overcome the immunosuppression.

(2) The immune system may also be destroyed by chemo or radiation therapy, and the anti-tumor response may decrease simultaneously. Thus, relapsed tumors easily escape the immune system.

(3) We agree with the reviewer that the relapsed tumors after chemo or radiation therapy often escape the immune system. However, our findings showed a better therapeutic effect by vaccination using inactivated slow-cycling tumor cells compared with normal tumor cells, which indicated a better immunogenicity of drug-treated tumor cells. Thus, whether the immune escape of relapsed tumors was caused by the low immunogenicity of tumor cells survived from chemo or radiation therapy still needs further study.
Track Changes:

1. The title “Immunotherapy using slow-cycling tumor cells prolonged the overall survival of mice bearing colon carcinoma” on page 1 has been changed to “Immunotherapy using slow-cycling tumor cells prolonged overall survival of tumor-bearing mice”.

2. Page 3, paragraph 1, line 1, “Despite a lot of progress has been made in the development of anticancer therapies, the mortality caused by the relapse and metastasis of cancer is still progressing” has been replaced by “Despite considerable progress in the development of anticancer therapies, the mortality caused by cancer relapse and metastasis is still increasing.”

3. On page 4, the keywords has been changed from “Slow-cycling tumor cells; Drug resistance; Cancer stem cell; Relapse and metastasis; Immunotherapy; Tumor vaccine” to “cancer relapse; cancer stem cells; drug resistance; immunotherapy; metastasis; slow-cycling tumor cells; tumor vaccine”.

4. Page 4, paragraph 2, line 1, “In the majority of cancer cases, the primary tumors causes 10% death of patients, while metastases formed by cancer cells separated from primary tumors are responsible for the main cause of mortality” has been changed to “In the majority of cancer cases, most mortality is caused by metastases, with only 10% being caused by the primary tumor”.

5. Page 5, paragraph 2, line 1, “In this study, we proved the higher tumorigenicity and drug-resistant potential of slow-cycling tumor cells” has been replaced by “In the present study, we demonstrated higher tumorigenicity and drug-resistant potential of slow-cycling tumor cells compared with normal tumor cells”.

6. Page 5, paragraph 2, line 5, “We reported that inactivated slow-cycling and drug-resistant cells induced more conspicuous proliferation and more IFN-gamma (IFN-γ) production of spleen cells in vitro” has been changed to “We showed that compared with normal tumor cells, inactivated slow-cycling tumor cells induced more proliferation of and more interferon (IFN)-γ production by spleen cells in vitro.”

7. Page 6, paragraph 3, ethics was added to the Methods section according to the
suggestion of editor.

8. Page 13, paragraph 1, line 3, “A bright DiI+ population of cells (DiI high) was visible after 8 days culture or 25 days growing in vivo (7.3% in vitro and 0.5% in vivo)” has been replaced by “A bright DiI+ population of cells (DiI-high) was visible after 8 days culture (7.3%, Figure 1A) or 25 days growth in vivo (0.5%, Figure 1B)”.

9. Page 13, paragraph 3, the percentages have been removed, because they seem unnecessary here.

10. Page 14, paragraph 2, the percentages have also been removed.

11. Page 15, paragraph 2, line 3 to line 8, the two sentences have been revised.

12. Page 16, paragraph 2, line 2, “Whereas tumor cells survival from chemotherapy were most DiI-retaining slow cycling cells (98.2%) compared with 2.8% of cells untreated, showed in Figure 4A and C.” has been changed to “Tumor cell survival after chemotherapy was then analyzed by flow cytometry. Most cells were DiI-retaining slow-cycling cells (98.2%), while there was 2.8% DiI+ cells in the untreated group (Figure 4A and C).”

13. Page 17, paragraph 3, line 9, “Then study of survival time showed that treatment with tCT-26 cells plus GM-CSF prolonged survival of tumor bearing mice obviously (Figure 6B).” has been replaced by “Treatment with tCT-26 cells plus GM-CSF prolonged survival of tumor-bearing mice compared with the other four groups (Figure 6B).”

14. Page 24, paragraph 1, line 4 to line 6, the two sentences were added to explain Ag repertoire change according to comment 4 of reviewer Yukai He.

15. Page 25, paragraph 1 was deleted because it appears to be repeated in the Conclusions section.

16. Page 26, paragraph 3, authors’ contributions were modified according to the editor’s suggestion.