Author's response to reviews

Title: Cellular immunotherapy using irradiated lung cancer cell vaccine co-expressing GM-CSF and IL-18 can induce significant antitumor effects

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Author's response to reviews: see over
Dear editor,

Thank you very much for processing our manuscript entitled "Cellular immunotherapy using irradiated lung cancer cell vaccine co-expressing GM-CSF and IL-18 can induce significant antitumor effects ". Comments and suggestions provided by the two reviewers have been very helpful to improve our manuscript quality and our study. We have responded to their constructive comments and suggestions point-by-point. Furthermore, the relevant revisions have been made in the original manuscript according to the comments of reviewers. Hope these will make it more acceptable for publication.

Thank you once again for your attention and consideration.

Sincerely yours,

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Response to the reviewers’ comments

Reviewer #1

The manuscript examines how using combination of IL-18 and GM-CSF gene to modify tumor cell vaccine inhibits tumor growth in vivo. It finds that it is mediated though CD4\(^+\), CD8\(^+\)T lymphocyte and this vaccine induces greater infiltration of spleen cells and higher production of IFN-\(\gamma\). The study is solid, with important new findings well supported and the manuscript is well written. There are, however several points that need to be addressed, as indicated:

Minor Essential Revisions

1. In background section, authors showed that IL-18 and GM-CSF had anti-tumor effects, why the cell viability didn’t have any change after LL/2 cells transfected with IL-18 and GM-CSF plasmids (24h – 72h)?

Our response: ******* Thank you for your comments. Although we showed that IL-18 and GM-CSF had anti-tumor effects in background section, the mechanism was mainly depend on immune responses in vivo. To our knowledge, these two cytokines have no direct antitumor effect in vitro.

2. Authors showed that transfected LL/2 cells were irradiated with a sublethal dose X ray (100Gy) to prepare vaccines, why the X ray dose was 100Gy? How about the other dose?

Our response: ******* As we know, melanoma cell engineered to secrete GM-CSF were irradiated under 100Gy X ray dose. (Vaccination with irradiated, autologous melanoma cells engineered to secrete granulocyte-macrophage colony-stimulating factor by adenoviral-mediated gene transfer augments antitumor immunity in patients with metastatic melanoma. Journal of clinical oncology 2003, 21(17):3343-3350.). In our preliminary study, according to the dose of melanoma we groped the irradiation dose of LL/2 under 50Gy, 100Gy and 150Gy respectively. The cell adherence and proliferation was evaluated under different dose using
morphological observation and MTT assay. We found that the LL/2 cell satisfied the optimized condition “no tumorigenicity but secreting cytokines” under 100Gy X ray dose. This is the best appropriate sublethal dose.

Reviewer #2

1) Figure 2A. Need to show GFP expression in control cells?

Our response: --------- Thank you your comments. Your suggestion is very useful for our manuscript. We have added the GFP expression in control cells as shown in our revised manuscript (Fig1A).

2) Figure legend in 2B is confusing what is the difference between the line versus the marker?

Our response: --------- There is no difference between the line versus the marker. This is due to our negligence and we have corrected it carefully. The result was showed in our revised manuscript (Fig1B).

3) Figure 3. The schedule indicates weekly measurements but the graphs of the tumor volumes show more than this occurred and dissection only occurred 11 weeks after initiation. Mice likely did not survive all 11 weeks in the control group thus it seems dissection may have occurred when a mouse died? What does the arrow at week 0 indicate? This section needs to be cleared up

Our response: --------- We understand that the misunderstanding might be caused by the unclear description of schedule. The schedule didn’t indicate weekly measurements, and was only a representative of measurement from week 6 to week 9. At week 6, the tumor volume was measured every 3 days for 6 times until week 9. The graphs of the tumor volumes also show that we have measured the tumor volume for 6 times in total. Mice in control group were all died at 45 days after inoculation, and the tumor was dissected when the mouse died. Week 0 indicated that mice were purchased. After 1 week, mice were immunized with vaccines.

4) Prophylactic immunotherapy in vivo: This section is confusing and needs revising. "About one week, tumor volume could be measured for six times every three days."
This sentence has no meaning that I can understand. Were tumors measured every three days and if so, what does 6 times refer to? The number of measurements taken for each mouse?

Our response: --------- This is our mistake for confusing the measurements. We have corrected this sentence in our re-manuscript. The right sentence is “About one week, tumor volume could be measured every three days and each mouse was taken for measurements. We measured for six times in Prophylactic immunotherapy”

5) Why are there such great differences in the size of the tumors at 23 days from the prophylactic versus the adoptive transfer experiments in the control cells (ie LL/2)? On days 23, the prophylactic experiments were 1500 but in the adoptive transfer they are 3000?

Our response: --------- The difference between prophylactic and adoptive transfer experiments might due to the fact that mice in the control group of prophylactic experiment were immunized with serum free medium and mice in adoptive transfer were received normal splenic lymphocytes. These differences may affect the speed of tumor growth. We think that some other factors might also contributed to the great difference between prophylactic and the adoptive transfer experiments, such as the state of cell, state of different batches of mice.

6) Figure 3C: How is death determined in these mice? In the combined IL-18/GM-CSF vaccinated mice, they must have some tumors as there is tumor volume but they are alive. What is the criteria used for this analysis to say a mouse is alive? Are they killed when the tumor size reaches a certain size and if so what is that size?

Our response: --------- The criteria used to analyze a mouse alive is that the tumor volume is not exceeding 1500mm$^3$. When it exceeded the criteria, the mouse could be default for death.

7) Figure 6B appears quite misleading. The small red dot in the cd8 panel for irradiated ll/2 appears to be what the authors believe is a CD8 cell, however T cells stained for surface markers make a circle around the cell. This appears to Be nothing more than an artifact. Looking at the other panels, all of the "positive" cells appear to
be just that, artifacts (especially what may have been called an NK cell in the mIL-18 NK panel." If these are not what the authors are including as positives, they need to show the panels where there truly is a cell that stains positive and actually looks like a leukocyte.

**Our response:** In order to confirm a leukocyte that stains positive, we have chosen some positive signals and made the DAPI alone, CD8 alone and merged pictures available. We thought the cell marked by white arrow looks like a positive leukocyte.

![Cell marked by white arrow]

8) The TUNEL staining in Figure 7 also is difficult to judge whether or not these are nuclei that stain positive. I recommend making the DAPI alone, TUNEL alone and merged pictures available. Some of the TUNEL nuclei are minuscule. And although apoptosis does induce pyknotic nuclei, what were the parameters to call a nucleus positive? It looks like there is definitely more TUNEL staining, however what is actually being called a positive nucleus is nebulous.

**Our response:** Your suggestion is very useful for our manuscript. We have made the DAPI alone, TUNEL alone and merged pictures in our revised manuscript (Fig6C) according to your recommendation.

Minor Essential Revisions

1. Figures 1, 2C are best served in supplementary data sections.

**Our response:** We have put Figures 1, 2C as supplementary data in our revised manuscript.

2. Why the need to show normal LL/2 vaccination did not work when you have the irradiated control? Did these non irradiated cells also grow in the mice?

**Our response:** We understand that the misunderstanding might be caused by
the unclear explanation of groups in our manuscript. As we known, some tumors have strong immunogenicity and can cause antitumor immune responses as the whole tumor vaccine. In order to eliminate its strong immunogenicity, we need to have irradiated control group in which mice were immunized with irradiated LL/2 tumor cell. The normal LL/2 vaccination as control group which only receive serum free medium alone did not work when compared with the irradiated control, further suggesting the irradiated LL/2 with low immunogenicity could not cause strong antitumor response. These non-irradiated cells can grow when inoculated with mice.