30133 Revised versions

Dear Editor Yuan Qi,

Thank you very much for this opportunity to improve our paper titled as "Dendritic cells engineered to secrete anti-DcR3 antibody augment cytotoxic T lymphocyte response against pancreatic cancer in vitro". Now, we are resubmitting this paper to WJG.

Generally, there were three reviewers' comments. According to these insightful comments, we have carefully revised the paper. All revisions were highlighted by yellow.

Thanks again.

Best wishes,

Jiang Chen

Response to 03471272 reviewer's comments

Comment 1- The writing often lacks clarity and sharpness, and the Results and Discussion sections are poorly organized. The authors should remove ambiguities, repetition and confusion.

Answer: Thank you very much for your comments. We have revised our manuscript carefully and reorganized the results and discussion sections according to the comments. Meanwhile, we have tried our best to remove the ambiguities repetition and confusion words in the paper.

Comment 2- It would be better to explain the underlying molecular mechanisms of weak antigenicity in pancreatic cancers.

Answer: Thank you very much for your comments. We have added the related contents, as follows. These words have been highlighted by yellow in the Introduction section.

Owing to lacking of expression of MHC class II molecules and co-stimulatory molecules, down regulation of expression of genes associated with antigen

presentation and low level of expression of tumour-associated antigens (TAA), PC displays weak antigenicity and high heterogeneity

Comment 3-The authors should explain how to obtain peripheral blood monocyte cells.

Answer: Thank you very much for your comments. We have added the related contents, as follows. These words have been highlighted by yellow in the Materials and Methods section.

(PBMCs), isolated by Ficoll-Hypaque (Sigma) density gradient separation, were used as the nonmalignant control tissues.

Comment 4- The authors should provide more detailed information about the pancreatic tumors and nonmalignant control tissues which they used for this study.

Answer: Thank you very much for your comments. We have added the related contents, as follows. These words have been highlighted by yellow in the Materials and Methods section.

(PBMCs), isolated by Ficoll-Hypaque (Sigma) density gradient separation, were used as the nonmalignant control tissues. Pancreatic cancer specimens were obtained at the time of surgery and were stored in RNAlate (Ambion Austin, TX) at 4°C until processing.

Comment 5- The manuscript would be improved if the authors confirm the effect of their method using dendritic cells obtained from several individuals.

Answer: Thank you very much for your comments. We have added the related contents, as follows. These words have been highlighted by yellow in the Materials and Methods section.

Fiveteen HLA-A2+ PC patients (9 males and 6 females, median age 53.5 years, range 35 to 72 years) were included in this study. Acorrding to the TNM classification of AJCC[21], there were 10 stage II patients and 5 stage III

patients. The location of tumor was divided into head (7 cases) and body/tail (8 cases).

As you known, pancreatic cancer (PC) is a deadly disease with a relative very short survival time. It is very difficult to obtain sufficient specimens of peripheral blood cells for the separation of the dendritic cells and going on the follow-up experiments in PC patients at same time complying with the requirements of the Ethics Committee. In fact, most of the patients have passed away before they could provide more specimens and these patients were excluded by our study regrettably at last. This is the main reason that why only 15 specimens included in our study. Indeed, Previous studies about PC dendritic cell vaccination found that specimens adopted by basic research were often less than 10 (J chen et al. 2015 exp bio med & Kalay et al, 2013 J gas sur). This is also what we would like to explain to the comments of the reviewer.

Comment 6- The authors described "over 90% of the cultured primary tumor cells showed a DcR3-positive expression" in Figure 1A, but it seems unclear.

Answer: Thank you very much for your comments. We have turned the description into "Most of the cultured primary tumor cells (over 90%) showed a DcR3-positive expression" to make the describe be more clear in the Result and Figure legend sections, Meanwhile, we improved the resolution of the Figure 1A to make the photo be more clear.

Comment 7- I suggest that the text about previous studies be moved from the Discussion section to the Introduction section.

Answer: Thank you very much for your comments. We have moved the previous studies parts from the Discussion section to the Introduction section, these words have been highlighted by yellow in the Introduction and Discussion section in the manuscript.

Comment 8-There are some typographical errors throughout the manuscript.

Answer: Thank you very much for your comments. We have tried our best to amend those typographical errors throughout the manuscript.

Response to 00502947 reviewer's comments

Comment 1- The English and scientific terminology throughout the manuscript needs major attention by the authors. The discussion tends to repeat what is in the Introduction.

Answer: Thank you very much for your comments. We have invited a native English-speaking professional to make a great deal of language polishing carefully and tried our best to amend the English and scientific terminology throughout the manuscript. Meanwhile, the repeat section in the Introduction and Discussion was be deleted. These words have been highlighted by yellow in the Introduction and Discussion section in the manuscript.

Comment 2-It is not clear whether the data presented are the mean+/-SD of triplicates of one experiment and this is representative of 3 experimental runs or the mean +/SD of three experiments. Please make this clear.

Answer: Thank you very much for your kind comments. We have outlined the repeat times of experiments in detail in the Method and Figure legend sections. These words have been highlighted by yellow.

Three representative experiments were rune, and the data are shown as mean ± SD.

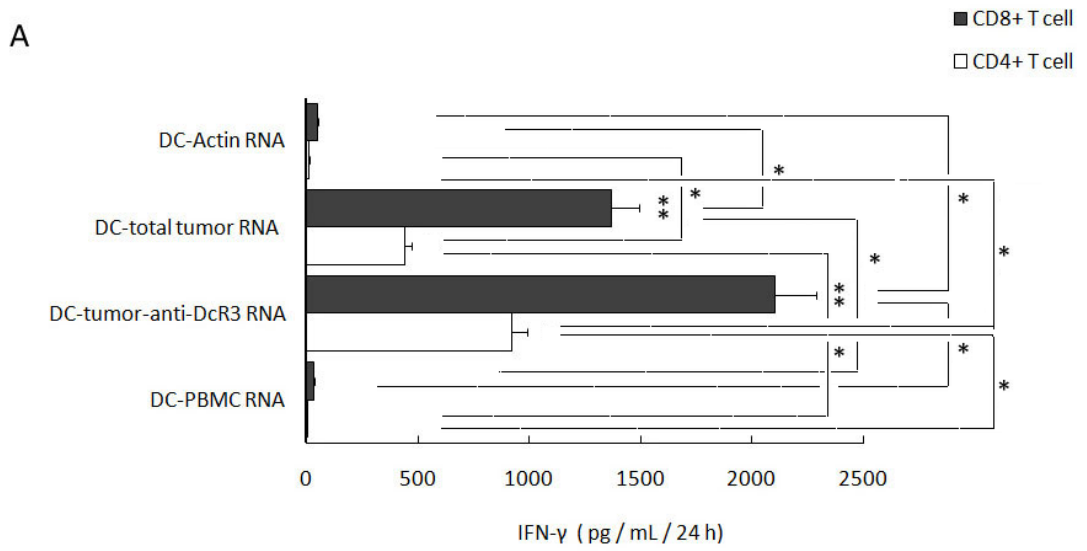
Comment 3-In the statistics it should be made clear which post hoc test was used?

Answer: Thank you very much for your comments. We have made clear that S-N-K post hoc test was used in the Statistics section. These words have been highlighted by yellow.

Statistical analyses, including ANOVA and post hoc test (S-N-K method), were performed

Comment 4-I find the number of * in Fig 5 confusing. Can you draw lines across the columns and place the star (*) on these to indicating what is being compared as per Fig3?

Answer: Thank you very much for your comments. Following you instruction, we have tried to draw lines across the columns and place the star (*) on these to indicating what is being compared in Figure 5, but we found it make more confusing just like the figure below, so we finally did not fix the figure 5. I am sorry.



Comment 5-The western blots in Fig 2A is not acceptable some of these lanes look as if they come from different runs, lanes then cut and collected into the presented figur. You should show a blot which been used to run these together.

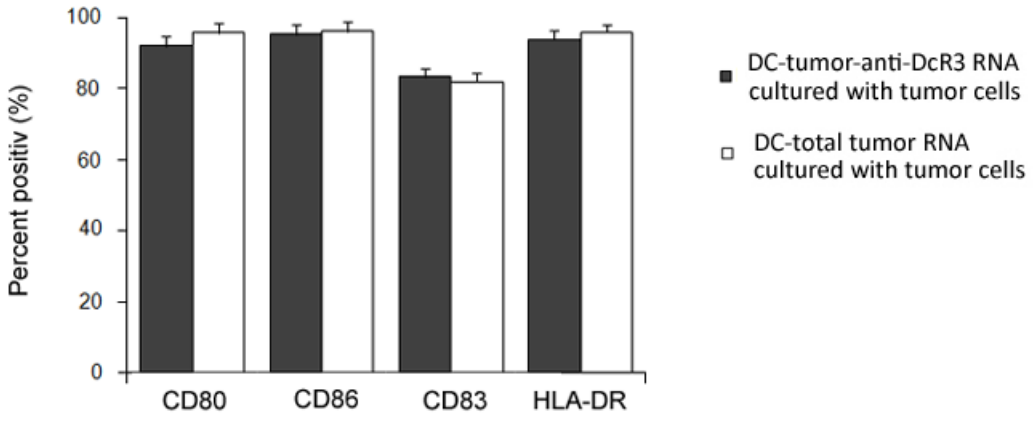
Answer: Thank you very much for your comments. We have redone the western blots experiment and took the place of former Fig 2A with a new one.

Comment 6-Fig 4: It looks as if there is a mistake in Fig 4A. Have the Total RNA vs RNA tumour-antiDcR3 antibody been mixed up?

Answer: Thank you so much for your kind comments. We did mix up the total RNA with the RNA tumor-anti DcR3 antibody, and we have corrected it in the Fig 4A. Thank you again!

Comment 1- DC are terminally differentiated cells, they do not proliferate, and short encounter with naive antigen specific T cells has been shown to be sufficient for optimum priming of CTL precursors. Authors claim that DC secreting anti-DcR3 antibody can generate superior anti-tumor T cell response. DcR3 has been previously shown to up-regulate of some co-stimulatory molecules but down-modulate others and modulate T cell response towards Th2 subtype (Hsu et al. Journal of Immunology, 2002). Therefore, they should include data showing co-stimulatory molecules and MHC class I and MHC class II molecules expression in DC with/without DcR3 expression.

Answer: Thank you very much for your insightful comments. In fact, we did test the function of DcR3 secreted by cultured primary PC cells towards DCs with / without the expressions of anti-DcR3 mAb. We found both the DC-tumor RNA and DC-tumor-anti-DcR3 RNA showed positive expression of CD80, CD86 (co-stuimulatorty molecules) and HLA-DR (MHC II molecules) after co-cultured with PC cells for 24h, but there were no significant difference between the two methodologies, as shown in figure below.



It was assumed that these results might be partly because a relative short encounter period (24h) between DcR3 and DCs. The result was not positive and we did not have enough understanding of its importance, therefore, we chose to ignore the related data about co-stimulatory molecules and MHC class I and MHC class II molecules expression in DC with/without DcR3 expression.

Now, following your instruction, we have added the related description and data in the Methods and Results section, Those words have been highlighted by yellow.

Phenotypic analysis of DC by flow cytometry

Fluorescein isothiocyanate (FITC)-conjugated mAbs were purchased from BD Pharmingen (San Diego, CA, USA). After three washes in cold PBS supplemented with 0.5% of bovine serum albumin (BSA), DCs were fixed with 2% paraformaldehyde in PBS. The following mAbs were used: FITC-anti-CD80, FITC-anti-CD83, FITC-anti-CD86, and FITC-anti-HLA-DR. The stained cells were analyzed using flow cytometry.

Meanwhile, both the DC-tumor RNA and DC-tumor-anti-DcR3 RNA showed positive expression of CD80, CD86, CD83 (co-stuimulatorty molecules) and HLA-DR (MHC II molecules) after coincubated with PC cells for 24h, but there were no significant difference between the two methodologies (data not shown).

Comment 3- (a). There is minimal effect on cytotoxicity by anti-DcR3 producing DC in Fig. 3A. (b). Authors should show data on quantification of CTL response generated, either by tetramer assay and/or by ELISPOT assay. (c). If authors claim of generating superior CTL response is true and it is minimal in quantitative terms, authors should re-stimulate primary CTL response generated under similar conditions as used for initial priming and see if they can generate superior antigen specific CTL response. Authors should also look for memory markers in such CTL. It has been shown that DcR3 can block apoptosis in Jurkat just like Fas-Fc antibody. Authors should make sure that the effect they are seeing in better cytolytic function is not because of blockade of AICD in CTL rather than better CTL response generation due to superior priming of CTL precursors by engineered DC. Authors should check AICD in CTL in presence and absence of engineered DC to rule that out.

Answer: Thank you very much for your professional and insightful comments.

(a) In Fig. 3A, left panel. DC-tumor-anti-DcR3 RNA was used as not only stimulator cells but also target cells (Heiser et al. Cancer Research, 2001) and there is relative max effect on cytotoxicity by anti-DcR3 producing DC compared with other DC groups. We realized that the former expression was easy to cause ambiguity so we

have added some account explains in the Results and Figure legend sections. Those words have been highlighted by yellow.

As shown in left panel of Figure 3A, DC-tumor-anti-DcR3 RNA was used as not only stimulator cells but also target cells, while DCs transfected with total tumor RNA alone or other autologous RNA-DCs were used as targets.

(b) In this study, we used ^{51}Cr cytotoxicity assay to judge the Induction of antigen-specific CTL responses. In fact, a lot of studies (Chen et al. exp bio med, 2015; Kalady et al. J gas sur, 2013 and Pan et al. Int J bio sci, 2010 and so on) used the same method to test the antigen-specific CTL responses induced by specifically DCs. Of course, we realized that showing data on quantification of CTL response generated by tetramer assay and/or by ELISPOT assay was very necessary and which can make the results be more reliable. We are Eagerly expect to take those important suggestions into our future study, thank you again for your kindly reminds.

(c) In this study, we used ^{51}Cr cytotoxicity assay to compare the capability of antigen-specific CTL responses induction among DC-tumor-anti-DcR3 RNA group, DC-tumor RNA group and other DC groups. These research methods were adopted with the support of a series of researches and published literatures (He et al. Oncol let, 2016; Benecia et al. J tran med, 2008; Chen et al. Autoimmunity, 2013 and so on). We think these research strategies are concise and effective. The reviewer suggested that we should re-stimulate primary CTL response generated under similar conditions as used for initial priming and look for memory markers in such CTL in order to see if they can generate superior antigen specific CTL response. These proposals are very valuable and we have learned that there were some obvious shortages in the aspect of study design. We are glad to adopt your advices in our follow-up studies. Thanks a lot for your kindly reminds.

The reviewer mentioned that "**Authors should check AICD in CTL in presence and absence of engineered DC to rule that out**" and we think the suggestion is very valuable. In fact, we did ignore the function of blockade AICD of anti-DcR3 mAb produced by DC-tumor-anti-DcR3 RNA in CTL. We are glad to take this important point in our follow-up studies. And now, we have added some additional explanations in the Discussion section which have been highlighted by yellow.

In addition, it has been known that DcR3 can block apoptosis in Jurkat cell lines just like Fas-Fc antibody [38]. A better cytolytic function might possibly because of blockade of Activation Induced Cell Death (AICD) in CTLs ran by DC-tumor-anti-DcR3 RNA rather than better CTL response generation due to superior priming of CTL precursors by engineered DCs.This point cannot be ignored and deserves a further study.

Comment 3- Figure 4A labeling might not be correct as the data contradicts what is shown in Fig. 4B.

Answer: Thank you so much for your kind comments. We mixed up the total RNA group with the RNA tumor-anti DcR3 antibody group in the Figure 4A label, and now we have corrected the label and made sure that there were no contradicts with what is shown in Figure 4B.

Comment 4-Authors show that CD8 T cells generated produce higher levels of IFN-gamma and CD4 produce higher levels of IL-4. Authors should include data showing complete cytokine production profile of CD4 T cells by including data showing IL-10, TGF-beta, IL-4 and IFN-gamma production in the same experiments. Dc3R has been shown to skew T cell response towards Th2 and Th2 biased CD4 T cells by producing IL-10 might inhibit CTL generation. Such data is essential to know engineered DC`s ability to generate superior CTL response in physiological scenario where CD4 and CD8 T cells are not separated.

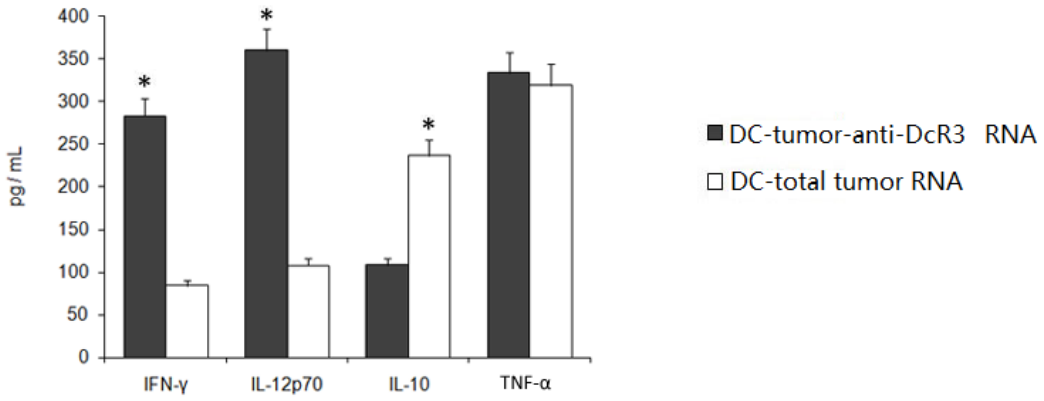
Answer: Thank you very much for your insightful comments. In fact, we did have analyses the panel of cytokines secreted by T cells co-cultured with DC-total tumor RNA and DC-tumor-anti-DcR3 RNA and **where CD4 and CD8 T cells are not separated. These cytokines were including** interleukin (IL)-12p70, interferon-γ (IFN-γ), IL-10, and TNF-α, as shown in figure below. **In order to reduce ambiguities, repetition and confusion of the manuscript, we deleted** these parts in the paper. Now, with the reviewer instruction, we have realized the importance of this part and added it back in the Methods, Results, Discussion and Figure5 legend. The words have been highlighted by yellow.

Analysis of cytokines released by T cells

As described by Miyazawa [28], after subjecting to different treatments, 5×10^3 DCs (DC-total tumor RNA and DC-tumor-anti-DcR3 RNA) were cultured in 96-well round bottom plates. T cells were isolated from proliferating peripheral blood lymphocytes and 5×10^4 T cells were stimulated with RNA-DCs in a total volume of 200 μL in 96-well plates for 24 h. The cytokines interleukin (IL)-12p70, interferon-γ (IFN-γ), IL-10, and TNF-α released by T cells were measured by enzyme-linked immunosorbent assay (ELISA) kits (Endogen, Woburn, MA, USA). The results were obtained from triplicate wells and the examination of supernatant from cultured T cells alone for the four cytokines were used as control groups.

T cell cytokine production pulsed by RNA-DC

Measurement of IL-12p70, IFN-γ, TNF-α, and IL-10 production by ELISA showed that there were no significant releasing of all the four cytokines in the culture supernatant of T cells (data were not shown). But T cells could secrete high levels of cytokines after pulsing with RNA-DC (Fig. 5A). The IL-12p70 and IFN-γ secreted by DC-tumor-anti-DcR3 RNA pulsed T cells were higher than those secreted by T cell pulsed by DC-total tumor RNA (*$P < 0.01$). No significant difference in TNF-α level was observed between DC-tumor-anti-DcR3 RNA group and DC-total tumor RNA group ($P > 0.05$). Simultaneously, the IL-10 level was lower in DC-tumor-anti-DcR3 RNA group than in DC-total tumor RNA (*$P < 0.05$).


400
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150
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IFN-γ
IL-12p70
IL-10
TNF-α
DC-tumor-anti-DcR3 RNA
DC-total tumor RNA