Author’s response to reviews

Title: Sub-lethal radiation enhances anti-tumor immunotherapy in a transgenic mouse model of pancreatic cancer

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PDF covering letter
Feedback to the reviewer:

Main concerns:

1. There is no indication of how the authors determine tumor incidence-size, histology, blood Glc?

   We have added the sentence "Rip1-Tag2 mice have 100% incidence of spontaneous insulinoma" in the "Materials & Methods" section. The tumor burden is determined with the measurement of cumulative burden volume. The calculation of tumor volume for each tumor is described in the "Materials & Methods" section: "volume = 0.52 x (width)^2 x (length)".

2. There is mention in the text that "In the regression trial? Activated splenocytes alone?" do not reduce tumor burden. However, the methods do not indicate pre-activation—this should be clarified as this is different from the figure legends.

   The splenocytes used in all the studies including the regression trials are obtained from naïve donors. The splenocytes do not go through pre-activation prior to transfer. We apologize for the miscommunication in the previous draft. The corrected sentence is: "...No reduction in tumor burden was observed following adoptive transfer of non-transgenic splenocytes alone".

3. The immunohistochemistry studies would benefit from quantification of the infiltrates. Also, in Fig 3, it is not clear from which group these sections were derived.

   Due to the commonly encountered variations in tumor infiltrates and immunohistochemistry, we feel that it would be difficult to provide a quantitative measurement of tumor infiltration. Rather, representative section for each condition is chosen to illustrate the differences observed with the treatment (radiation+TCR splenocyte vs each of the control conditions).

   The sections were derived from groups in the regression study at endpoint (16w). We have modified the description in the "Results" section to clarify this: "Tissue samples were collected three weeks after treatment in the regression trial".

4. The authors mentioned that CD34 staining does not indicate any alteration in vessel structure, but they do not mention vessel density.

   The CD31 (not CD34) staining has shown no significant alterations in either vessel structure or vessel density. We have included this observation in the "Discussion" section: "An analysis of the tumor vasculature in the combination trial by immunohistochemical staining for the endothelial cell marker CD31 revealed no obvious structural or density changes in tumor blood vessels (data not shown)".

5. There is insufficient consideration of the changes of infiltrates in terms of CD8 cells and CD11c cells. The authors should discuss the change in CD11c+ cells after irradiation (alone) as well as the infiltration of CD8 cells in terms of potential specificity. Similarly, the authors should consider/mention the effects of transfer of normal, non-transgenic splenocytes. Finally, all of the infiltrates are presumably considered of donor origin, so what is the activation profile of CD4+ T cells after transfer?
There is some increase in dendritic cell infiltration into solid tumor in the group that received radiation alone. We have included the observation and interpretation in the "Discussion" section: "Indeed, in the regression study, we observe some increase of tumor-infiltrating dendritic cells in the group receiving the single radiation treatment. It is possible that radiation-induced, tumor-infiltrating dendritic cells may facilitate sustained activation and infiltration of the adoptively transferred CD4+ T cells in solid tumors in the combination group".

It is possible that the non-transgenic, CD4+ T cells present during TCR splenocyte transfer can be induced to infiltrate solid tumors by transgenic (Tag-specific), CD4+ T cells. This may be part of a "bystander" effect. We have included this in the "Discussion" section: "Due to the nature of this line of TCR transgenic mice, only about 10% of splenic CD4+ T cells are Tag-specific. Thus, it is possible that some of the tumor-infiltrating, CD4+ T cells may recognize targets other than Tag. This may be the result of the "bystander" effects induced by the tumor-infiltrating, Tag-specific, CD4+ T cells. The elucidation of this phenomenon with idiotype-specific antibodies or tetramers in future experiments would clarify the extent of "bystander" effects in this model. Because the infiltration of CD8+ T cells is preceded by that of CD4+ T cells, we reason that the infiltration of CD8+ T cells is facilitated by the infiltrating CD4+ T cells. We have included this hypothesis in the "Discussion" section: "We speculate that the tumor-infiltrating, CD4+ T cells would induce the activation and tumor-infiltration of CD8+ T cells present in the transferred transgenic splenocytes."

We agree with the reviewer that it would be very informative to examine the activation state of the tumor-infiltrating, CD4+ T cells under different treatments. Yet, such analysis is incumbent upon having CD4+ T cells with the same antigen specificity. As mentioned above, due to the unique nature of our line of TCR transgenic mice, a large fraction of the transferred CD4+ T cells don't express the transgene. Thus, it is possible that the non-transgenic, CD4+ T cells of donor origin would be induced to infiltrate solid tumors by the transgenic (Tag-specific), CD4+ T cells, as part of a "bystander" effect. The assessment of the activation status of the tumor-infiltrating, CD4+ T cells has to be preceded by the purification of Tag-specific, CD4+ T cells with idiotype-specific antibody and/or TCR-specific tetramer. Due to the complexity of these experiments, the limited amount of lymphocyte material obtainable from the lesions, and the fact that disruption of T cell anergy/tolerance by radiation is only one of a number of possible underlying mechanisms implicated by this study, we feel that it would be more appropriate if these future studies are presented in a separate publication that deals on a more mechanistic level the effects of radiation on T cell activity and/or tumor microenvironment.

**Minor points:**

1. How many mice per group are used in the survival study?

   For intervention study: untreated control, n=12; 600R alone, n=10; TCR splenocytes + 600R, n=10; TCR splenocytes + 600R (x2), n=10;
   For regression study: untreated control, n=12; 600R alone, n=11; TCR splenocytes + 600R, n=16; C3H splenocytes + 600R, n=10; TCR splenocytes + 600R (x2), n=9.
   This information has been added to the figure legend.
2. Find a better way to say "Significant lifespan extension"? e.g., prolongs survival?
   We have made the changes as indicated by the reviewer.

3. What is the frequency of TcR transgenic T cells in the splenocytes?
   The frequency of TcR transgenic T cells in the CD4+ splenocytes is around 10%.
   We have added a sentence in the "Materials & Methods" section: "About 10% of the splenic CD4+ T cells in this transgenic line express the TCR transgene (28)."

4. Why was a Mann-Whitney test used? Does this consider standard deviation as in some cases, the error bars suggest lack of significance?
   The distribution of the data dictates the choice of T tests. In this case, the appropriate T test is Mann-Whitney (for non-parametrically distributed samples). We used Instat Version 1.12 (GraphPad Software) for the statistical analyses. This information has been added to the "Materials & Methods" section. Due to the nature of the sample distribution, the standard deviations can be larger than those in normally distributed samples. However, the appropriate T test indicates highly significant statistical differences.