Author's response to reviews

Title: Evaluation of Immunological Escape Mechanisms in a Mouse Model of Colorectal Liver Metastases

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Author's response to reviews: see over
Dear Dr. Alam,

First of all the authors would like to apologize for the delayed resubmission. We thank the reviewers for their constructive criticism of our manuscript. In addition we would like to express our gratitude for your encouragement to resubmit a corrected version of our contribution.

In the following we address the comments of the reviewers. The changes in the manuscript have been highlighted. Almost the complete Discussion has been rewritten; all graphs have been changed regarding their design. We have included the SD values and visualized the significances.

We would appreciate very much if you would consider this corrected version for re-evaluation as an original article for publication in *BMC Cancer*.

Yours sincerely

Andreas Thalheimer, M.D.
Reviewer #1:

1. p 8 l24: Normal liver tissues used as “naïve controls” need to be described in greater detail: were these animals comparable to those used in the experiment (age/weight/sex/strain - any pretreatments?)

The control liver tissue was obtained from balb c mice of the same age, weight and sex than all other animals analyzed. The control animals were surgically treated with a laparotomy and an intraportal injection of PBS without tumor cells. This has now been mentioned in the Methods section (surgical procedure).

2. p8: A description of how the percentage of immunohistochemically stained cells was determined is lacking and needs to be added to the method section. Likewise, the evaluation of immune-fluorescences should be described.

On p8 in the Methods section the description how the quantification of immunohistochemistry and likewise immunofluorescence was determined has been added in the revised version.

3. p11: RT PCR: How was amplification of genomic DNA excluded?

On p8 we now have added how genomic DNA was excluded.

4. p13 l19: It is stated that seven animals per group were used for RT experiments. However, in the statistics section of the methods, it is described that the analysis was performed on five animals per group. This needs to be clarified. Also, it does not become clear why animals at day five after injection were not included in the analysis. These data should be presented as well. See also p12 l20.

Thank you for this comment. We had seven animals in each group and three animals in the control group. This has been corrected. Additionally we have included the data of all animals on day 5 after injection in all analyses. The data are shown in the different graphs.

5. Figures 2 - 4: SD and ranges should be provided in the Table below each Figure. For all Figures, it has to be indicated (e.g. by a line) which comparisons exactly were found to be significant. Values for day 5 post-OP need to be presented.
We have changed the graphs compared to the original submission. Now, each surface antigen and each cytokine is grouped with the changes over time. The SD is now included in the tables below the graphs. We did not include the ranges because this additional information confused the illustration. What we did, however, was to include the statistically significant differences into the figures by adding a line which indicates significance.

6. Figures 2C, 4D: The proportion of immunoreactive cells appears to be very high. Moreover, the single staining for CD8 (Figure 4D, middle upper panel) seems to have a nuclear component. This might be attributed to thickness of the specimen or non-specific background staining. The authors should provide conventional IHC single stainings for CD8 and CD4 in order to give a better impression of T cell infiltration. This would also help to judge on histology of the stained tissue. Unfortunately, the dotted lines and asterisks provided are not helping to that end (asterisks being truncated, too).

Indeed the proportion of infiltrating T cells is—compared to the human situation—very high. We have discussed this matter on p14 now. Additionally, a conventional IHC with double staining of CD4 and CD8 is displayed as figure 2a, showing the increase in immunoreactive cells from day 10 to day 20.

7. Figure 2B: From the table provided it looks like about 59% of cells were T cells (34% CD4+ and 25% CD8+) in the tumor at day 20 after tumor cell injection. This number appears very high. IHC stainings need to be provided (see also comment to Figures 2C, 4D) and this finding needs to be discussed.

See also 6.

8. Discussion: A large proportion of the discussion is speculative. The authors need to be careful about the lines of argumentation used and should keep in mind that their results are (i) observatory and (ii) derived from an artificial animal model. Parts of the discussion should be rewritten and toned down.

This is a very important comment. After careful re-reading of the Discussion section and intensive discussion in our working group we decided to completely re-write the Discussion section. We think that now the findings in our study are conclusively discussed with the current literature.

Additionally we now have discussed the limitations of the animal model we used in this study (p14).
Minor essential revisions:
1. Basic and well established mechanisms of immune evasion like for example alterations in the cellular antigen processing and presentation machinery (loss or downregulation of MHC I, downregulation of APM components) have not been studied. The authors might want to comment on classical mechanisms of immune evasion in introduction and discussion.

Thank you for this comment. We now comment on classical mechanisms of tumour immune escape in our Introduction section, which partly has also been rewritten

2. Abstract p2 l17, l18: The expression “stage-dependent” is misleading and should be replaced.

This has been corrected.

3. p10 l10: The authors state that they analyzed six HPF representative of the whole tumor section. However, on p8 l6 it reads like biopsies were only taken from the margin of the metastasis. Given the known heterogeneity of lymphocyte infiltrates between margin and center of metastasis, the authors might want to comment on that.

This is indeed capable of being misunderstood. We corrected this on p6. Half of the liver tissue was fixed in formalin, the rest divided into snap-frozen tissue for RNA-analyses or Acetone for cryostat sectioning.

4. p10 l11-12: The sentence needs to be rewritten.

This has been changed with corrections highlighted.

5. p13 l5-9: The description of injection with 1x106 tumor cells does not add to the results.

We now have removed the description of injection with 10^6 tumor cells

6. p13 l26 “increasing” should be replaced, because biopsies from different animals were used at different points of time.

“Increasing” has now been replaced by “higher level of” (p 11).
7. **Figure 1**: SD of tumor volume for time 5 days is lacking. The statement “in all animals” should be omitted.

Figure 1 has completely been removed upon the recommendation of reviewer #3

8. **Figure 2a, 4a**: “Metastatic liver” should be changed to “metastatic tissue”.

This has been changed with corrections highlighted.
Reviewer #2:

1- The authors show that over time (up to 20 days) the tumor volume increases 5 times between day 10 and day 20. Therefore it is not surprising that also the number of TILs of different nature increases. For a better representation of this infiltration the authors stain the tissues with antibodies characteristic for certain subpopulation, CD4, CD8, CD25 and FoxP3 and suggest an increase of all four populations. Unfortunately, they only show CD4 and Foxp3 in Fig2c. While an increase in FoxP3 between day 10 and day 20 is visible, no difference can be detected between CD4 day 10 and CD4 on day 20. According to the picture given in the manuscript it remains unclear how counting of the cells could be done properly and it would be of interest to see, how the TIL proportion varies in primary (human) tumor samples of different size.

We agree that a single figure with CD4 and Foxp3 is not sufficient to demonstrate an increase of all T cell populations. We therefore have added an additional immunohistochemical figure demonstrating an increase of CD4+ and CD8+ T cells from day 10 to day 20 (fig. 2a). Regarding the counting of immunohistochemical staining see comment to reviewer#1, 2.)

2- The same figure identifies the FoxP3 population as CD4 helper T cells, staining with CD8 and merging the pictures would have been an appropriate control, which however is missing.

It is absolutely right that both in humans and in mice there is a small population of Foxp3+CD8+ effector cells which make up for just a very small portion of CD8+ T cells. We are aware of the discussion that Foxp3 is not necessarily a specific lineage marker for Tregs but is linked more to functional suppression. The result seems to be the same even if we could detect some CD8+Foxp3+ cells in our samples: the more Foxp3+ cells the more protumoral immune reaction.

3- The potentially increased infiltration of T cells is attributed to an increased level of cytokines namely IL-10, TGFβ and TNF. While this seems to be true for IL-10, the increase in TGFβ and even more in TNF is extremely weak. This is confirmed via immunohistochemical analysis of positive cells. Counting of cytokine positive cells is extremely critical because cytokines are released and cannot be attributed to individual cells on tissue any more. Such an analysis requires proper co-staining of tumor cells and/or TILs and intracellular
cytokines as well as highly preserved tissue architecture. None of these conditions were used, therefore it is unclear how reliable these data are.

Thank you for this comment. It is right that the immunohistochemical quantification of the named cytokines is problematic. Nevertheless the RT-PCR data were clear and could be confirmed by our IHC data. As we state in our discussion section now, IL-10 and TGF-β are released by Th2-cells and show an immunosuppressive effect. TNFα most probably is released by macrophages and dendritic cells and supports the development of IL-10 producing Th2-cells. This is now discussed on p16 of the Discussion section. In this study we do not try to answer the question which cells do release the single cytokine, we just wanted to determine the changes of the released cytokine level during carcinogenesis. But reviewer#2 is absolutely right to comment that an immunohistological allocation of the released cytokines to specific immune cells is very difficult.

4- Similar problems occur with the observation of FAS and FASL deregulation. The counterattack theory is discussed in a highly controversial manner, which is in part due to the fact that it is hard to prove in vivo. The observation by Grimm et al. that FASL increases, and it increased in tumor cells, while FAS receptor declines indeed supports this view. But, due to the fact that on the same tissue (Fig 4c) only FASL is shown but not FAS, is again a lack of support for the conclusions the authors draw in the results and discussion section. Why did they not stain FAS? The PCR data suggest a decline and the authors show tumour tissue with adjacent normal liver? This is similar important for Fig 4d, here the authors show a co-staining of CD8 plus cells, assumed to express FAS. This is very hard to see, the better magnification given in the lower panel would also have been beneficial for the upper panel.

Thank you for this comment. Since we rewrote almost the complete Discussion section the paragraph dealing with the counterattack hypothesis has also been corrected (p16/17).

Actually we did FAS staining in our tissue samples and found a decreased expression in liver metastases. However, we decided not to show this staining as a photograph, since we think that we do not want to overload the reader with pictures and graphs. The results of the staining is -we hope the reviewer agrees with us- demonstrated sufficiently.
Reviewer #3:

1- Materials and Methods are very detailed and should be shortened: For example culture of CT26.WT in pages 6 & 7; surgical procedure are well described in authors’ previous paper (reference 24). Also there are many unneeded details in other sections such as RNA extraction and real time PCR.

The Section “Methods” has been revised according to the suggestions of reviewer#3.

2- Results are generally not well described. Comparisons are not clear and p values should be indicated for every comparison as some differences are not significant such as CD25 at day 15 (figure 2a), TGF-b and TNF-a at day 15 (figure 3b), and FAS at day 15 (figure 4a). In figure 2a: Are authors comparing days 10, 15 and 20 to normal liver tissue? They should state that clearly in legend. Why Asterisk in figure 2b indicated on naïve? Why in figure 4b, no immunohistochemistry results for naïve are shown as for figures 2b & 3b?

We have completely revised all graphs and changed the presentation. Data are shown with SD; significances are presented as a line with asterisk. The results are presented in detail in the legend of each revised graph.

3- It is not clear why authors show immunofluorescence data in figures 2c & 4d and all data presented in these two figures are from immunohistochemistry analyses. Alternatively, some immunohistochemistry photos as representative examples should be shown in figure 2.

This is right. In the revised version we have included an immunohistochemical double staining of CD4 and CD8 (fig. 2a) comparing the T cell infiltration on day 10 vs day 20.

4- Discussion is not very much related to the data shown in this study. The authors can discuss their results compared to other studies (e.g. reference 4). In page 16, the statement of “Thus, our results support the position that defective anti-tumor cytotoxic T cell responses contribute significantly to the suppression of an anti-tumor immunological surveillance” is not supported by the data presented here as no anti-tumor immune response was evaluated. In page 17, the statement of “We demonstrated that CD4+CD25+ T cells and CD8+ cytotoxic T cells were attracted by cytokines and chemokines as well as other factors as a part of the tumor microenvironment” is not demonstrated at all in this work.

The complete Discussion section has been rewritten, see also comment 8, reviewer#1.
5- There is a problem with references and authors have to check their relevance carefully: For example in page 6 (reference 23), CT26.WT was not used in this study. In page 4, authors refer to one of their papers (reference 9) for inducible Treg, there are more relevant references. Similarly, reference 11 is not relevant.

We apologize for this mistake. Additionally we have checked all references for relevance.

**Minor Essential Revisions**

1- *Was the model used xenomodel as described in the background of abstract?*

The term Xenomodel is not correct in this context since we do not transfer human cancer cells into a rodent in the meaning of a xenotransplantation. We avoided this term in the revised version.

2- *In introduction, page 4, inducible Treg cells secreting IL-10 are Tr1 and not Th2.*

This has been corrected both in the Introduction and in the Discussion section.

3- *Figure 1 is a very simplistic one and is not needed as data are described in details in page 13.*

We have removed this figure.

4- *Day 20 on the photo of figure 2c should be made more clear.*

The message of this figure, which is figure 2b in the revised version, is that the number of Foxp3+ cells increases during tumor growth (i.e. from day 10 to day 20). We still think that the figure is properly demonstrating this situation. Of course it does not provide morphological information like an IHC staining. Nevertheless the immunofluorescence technique is a sufficient method for showing changes in cell quantity. A greater magnitude would also not help to get more information. We hope, reviewer#3 can connive at this argumentation.
5- Minor errors: In abstract, FOXP3 should be changed to Foxp3. Page 7: of all animals were (not was). Page 9: Albumin. Page 9: Fluorescein isothiocyanate looks German! Page 16: TGF-b and not anti-TGF-b.

Thanks for these corrections.

Fluorescein isothiocyanate is half-German, half English. We have corrected all of the above mentioned.