Author's response to reviews

Title: Use of a recombinant S. typhimurium strain expressing C-Raf for protection against C-Raf induced lung adenoma in mice

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Author's response to reviews:

PD. Dr. Ivaylo Gentschev

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Dear Editor,

we would like to thank the reviewers for their constructive review of our manuscript. Their suggestions were very helpful and we have addressed all issues and concerns raised and improved our manuscript accordingly. We hope that you now will find the paper suitable for publication in BMC Cancer.

Please find enclosed the point to point replies to the reviews.

Sincerely yours,

Ivaylo Gentschev

Reviewer: Carlos Alberto A Guzman
1) Rational for using i.v. boost

We agree with the reviewer that vaccination via the oral route primarily induces a mucosal immune response, whereas i.v. immunization results in a systemic immune response. For an effective immunotherapy, especially of tumors residing at mucosal tissues such as the lung tumors in our model system, a broad immune response encompassing both, the mucosal and systemic branch would be desirable. For this reason, we combined oral immunization with an i.v. boost within the experiments carried out in the BxB23 transgenic animals. This point was clarified within the manuscript as suggested by the reviewer (page 5).

The two different protocols for i.n. vaccination were applied, because we used two different transgenic cancer mice models (BxB23 and BxB11), which develop lung adenomas after different latency periods. However, the doses of immunization (4 times with 1x10^7 bacteria or 3 times with 1x10^8 bacteria) with regard to the amount of C-Raf antigen are relatively similar.

2) Results of parallel oral and intranasal vaccination

We do not agree with the reviewer's notion that we failed to show results for both vaccination routes in parallel, e.g. antibody responses of both immunizations approaches were demonstrated in Fig.3. However, a Raf specific CD8+ T-cell response was only observed in mice after an oral/intravenous immunization with SL7207/pMOhly-Raf. Therefore we did not present a common figure with results of this experimental setting.
3) Western Blot analysis

We have now included a Western Blot analysis (endpoint titration) as a supplemental figure. In this blot the preimmune serum and the serum of mice immunized either p.o./i.v. (Supplementary figure A) or i.n. (Supplementary figure B) with SL 7207/pMOhly-Raf were used in a dilution of 1:1000 on purified C-Raf-GST fusion. Both sera recognized C-Raf. However, a 1:2000 dilution of the same sera was not able to detect the same C-Raf band (data not shown).

Indeed C-Raf specific antibodies were detected in 20% of the vaccinated animals. We agree with Dr. Guzman that this result is not optimal, but for us the important information was to demonstrate that it is possible to induce a humoral immune response against C-Raf. The development of improved vaccination protocols and new kinetic studies for both immunization routes are currently carried out, but will need a significant period of time.

4) Passive transfer?

The suggested method of passive transfer by the reviewer is a good tool for the analysis of a protective role of antibodies in an infection mouse model. However, in our spontaneous tumor mice models, it is not really clear how long the C-Raf specific antibodies must be present in order to be protective. Therefore, we can not examine the role of the antibodies in the observed protection so fast.
We did not detect C-Raf specific CD8+ responses in C57BL/6 or BxB23 mice after an i.n. immunization with SL7207/pMOhly-Craf. This is addressed in the revised manuscript (page 9).

In the case of the BxB11 mice model (Fig.6) we have chosen the intranasal route of immunization, because in BxB23 model we found the best protective effect after an immunization via this route (Fig.5).

6) classical CTL tests

We did not perform classical chromium release assays for the evaluation of the CTL response. However, we and others have already shown that, except in some cases, data obtained from intracellular cytokine staining or ELISPOT analysis are comparable and more quantitative than chromium release data as there is no obligatory 5 day restimulation period (Fentslerle et al., J Immunol. 1999;163:4510-8). Although we can not formally exclude the possibility, that a chromium release assay would yield more stringent results with lower background values in our system we believe that the background problem would persist in this assay.
The quality of Fig.1 was improved

8) Nomenclature

Has been changed as requested by the reviewer

9) The age of the animals

This information has now been incorporated (page 5).

Reviewer: Thomas Rudel
We agree with the reviewer's concern that the tissue-specific expression of truncated human C-Raf in mice combined with a vaccine based on human C-Raf might not reflect the setting anticipated in humans. On the other hand, the human C-Raf has an identity of 94% to the mouse C-Raf. Therefore, the question of the efficacy of our vaccine in humans can be finally solved only in a clinical setting.

Autoimmunity not only depends on the presence of the antigen, but also on its expression level and the location/nature of the affected tissue. In the mouse model, BxB C-Raf is constitutively expressed in the lung tissue, but in tumors there is a marked increase of the expression level, which is also typical for many human tumors. Despite this ubiquitous expression within the lung, vaccinated animals showed no apparent difference concerning respiration related health status or histological signs of inflammation in non-affected areas of the lung. The reason for this might be that the levels of MHC class I presentation of BxB C-Raf derived antigens is below the threshold for efficient T-cell activation. On top of that, non-tumorous tissue might lack the proinflammatory signals needed for the homing of activated T-cells, which is a prerequisite for an efficient attack.

Concerning peripheral tolerance, we don't agree with the reviewer's opinion. Although peripheral tolerance might differ in an experimental system with pure lung-specific expression of the antigen compared to the human distribution of C-Raf expression, the transgene is already present at birth. As the lung tissue is not thought to be an immunoprivileged site, where the contact of T-cells with tissue specific MHC associated antigens is unlikely, peripheral tolerance can occur. However, as discussed above, expression levels might be below the threshold level needed for the induction of peripheral tolerance. Concerning central tolerance due to negative selection of autoreactive T-cells within the thymus, the group of Kyewski has already demonstrated that expression of virtually all genes examined, including so called cancer testis antigens, can be detected within the thymic stroma (Nat Immunol. 2001, 2: 1032-9; Nat Rev Immunol. 2004, 4: 688-98). In this context it is important to note that we were able to detect B-Raf specific antibodies in melanoma
patients pointing towards missing central tolerance and restricted peripheral tolerance (at least concerning B-Raf specific CD4+ T-cells) in melanoma patients (Fensterle et al. BMC Cancer. 2004 4: 62). Furthermore, we and others have already demonstrated in different experimental settings that peripheral tolerance and even CD4+ CD25+ T-cells mediated T-cell suppression can, at least partially, be overcome by bacterial vaccines (Kusar, et al., J Exp Med. 2002, 196: 1585-92).

In our study we did not compare the lung weight of wild type mice to the BxB-mice used in our studies. However, such an analysis was already done by Kerkhoff et al., Cell Growth Differ. 2000 Apr;11(4):185-90. The data showed an 8-10-fold increase in lung tissue mass compared to wild-type mice.

The two different protection assays were carried out only one time, but the data are statistically significant.
BxB11 and BxB23 develop lung adenomas after different latency periods. Therefore, we used BxB11, which has a shorter latency period compared to BxB23, for survival assays and BXB23 for the evaluation of the effect of the vaccine.