Author's response to reviews

Title: The mannosylated extracellular domain of Her2/neu produced in P. pastoris induces protective antitumor immunity.

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

We hereby submit for publication a revised version of 1923754115263936. We have carefully considered the constructive criticism of the reviewers and address all theirs comments. A point-by-point response to all issues raised by the reviewer is provided below. Except from the revised manuscript we provide a marked-up copy of the changes made from the previous article file as a SUPPORTING INFORMATION file. We thank you very much for your attention to this work and hope that you will find the revised manuscript suitable for publication in BMC cancer.

Yours sincerely,

Dr Avgi Mamalki
Response to Reviewer 1: Ai-Li Shiau:

1. “In this study, the ECD of human Her2/neu protein was used as the immunogen to induce antitumor immune responses in mice against syngeneic tumors artificially expressing exogenous human Her2/neu to mimic Her2/neu-overexpressing human tumors. As human Her2/neu protein is a foreign antigen to the mice, it is much easier to elicit potent immune responses in mice against the human Her2/neu antigen expressed on mouse tumor cells. This issue should be thoroughly discussed.”

The issue was thoroughly discussed and the following paragraph was added in the Discussion section (page 11). 6 more references were also added in the text.

“Negative selection in the thymus is capable of deleting almost all self-reactive T cells [25, 26]. However, because this process is not complete, several peripheral tolerance mechanisms persist [26]. Because most known tumor antigens, including Her-2/neu, come from self-proteins, these incompletely removed self-reactive T cells must be activated before being applied to tumor immunotherapy. Recently, several reports have shown that xenogenic vaccination could break self-tolerance and inhibit the progression of established tumor in syngeneic or transgenic mouse models [27-29]. In our study, xenogenic immunization with recombinant human ECD/Her2 most likely induced cross-reactive CTL against murine HER-2/neu which rejected the transplantable tumors expressing murine HER-2/neu. Murine endogenous T cells that specifically recognize the mouse HER-2/neu-MHC I complex might be high-avidity T cells. Such high-avidity T cells are eliminated in the thymus during the negative selection process due to autoreactivity or exist in the periphery in an anergic state. In contrast, low-avidity T cells induced by xenogenic ECD/Her2 are likely to recognize the altered peptide sequences of murine HER-2/neu, allowing the T cells to escape central tolerance and to remain intact in the periphery. Given that ECD/Her2 is a potential source of immunogenic determinants recognized by CD4+ T helper cells [30], it is obvious that activation of the latter cell population will also lead to generation of B cell clones producing anti-HER-2/neu antibodies, thus overcoming any tolerance mechanisms affecting the B cell compartment.”

References:

2. *The authors described the enhanced immunogenicity of mannosylated Her2/neu protein in the induction of immune responses. Although this may be true in the Her2/neu vaccine, the non-mannosylated Her2/neu produced from E. coli should be tested side-by-side with the mannosylated protein for their immunogenicity and protective immunity against Her2/neu-overexpressing tumors.*

The vaccination with the extracellular domain of the human Her2/neu receptor expressed in various transfected cell lines (DHFR/G8, BHK/erbB2, L cells etc) was previously extensively studied and it was shown that when these proteins were used as immunogenic molecules, they have no effect in reducing tumor growth. Given that these expression systems confer normal mammalian glycosylation, it is clear that the enhanced immunogenicity as well as the delay in tumor development caused by ECD/Her2 vaccination in mice, comes from the increased N-linked glycosylation of the high-mannose type of this molecule. These results were mentioned in the Background and Discussion sections of this manuscript.

3. *In Figure 2, mice immunized with ECD/Her2 for three times at days 0, 21, and 42 developed anti-ECD/Her2 antibodies. However, similar antibody levels were induced regardless of the numbers of immunization. Furthermore, at days 62 and 82, the background level of non-specific binding in the ELISA of the samples from the control mice was high. These points should be discussed.*

In order to show that the levels of anti-ECD/Her2 antibodies do arise depending on the number of immunizations, we have replaced figure 2, using a higher dilution of sera. This figure shows the ELISA assay that was performed using sera from the two groups of immunized mice at a 1:1000 dilution. The levels of anti-ECD/Her2 antibodies detected after the first immunization (day 21) were increased by two times after the first boost (days 42) reaching a plateau. These mice were challenged (day 52) by s.c. injection into the left flank with D2F2/E2 tumor cells, and this was noted in figure 2. The increased background level in the control mice (PBS) at day 82 is due to the Her-2/neu molecule that is expressed in the surface of D2F2/E2 transfected cell line, and this remark was added in the Results section (page 8).

4. *The authors claimed that the immunized mice did not elicit any self-toxicity compared to PBS-vaccinated mice without showing any data. As anti-Her2/neu antibody has been shown to exert some side effects, this issue should be addressed.*

In the Results section (page 8) the phrase “Mice weight was measured as an indicator of toxicity caused by vaccinations and no differences were obtained compared to PBS vaccinated mice (data not shown)” was added.

5. *In the animal studies, tumor-free survival of BALB/c mice bearing D2F2/E2 (n=9) was shown (Figure 4), whereas tumor volume of HHD mice bearing ALC/neu tumors (n=4) was shown (Figure 7). For better presentation of the efficacy of ECD/Her2 vaccine and the consistency of the data, both tumor volume and survival in*
the two tumor models should be shown. Furthermore, in Figure 7, the numbers of the mice per group should be increased.”

Figure 7 was replaced by a Kaplan-Meier representation of the results. To confirm our results obtained with Balb/c mice, we used HHD mice as a secondary animal model. Given the lack of a sufficient number of these mice in our animal facility, their number was lower.

6. “Figure 5 showed the immune serum from ECD/Her2-vaccinated mice could reduce the proliferation of SK-BR-3 cells. However, the degree of cell growth inhibition was not 13%. Therefore, whether the antibodies contained in the sera from ECD/Her2-immunized mice have significant anti-proliferative effects in SK-BR-3 cells cannot be confirmed.”

We believe that this point has been addressed in our results. Indeed, figure 5 shows that the diluted sera (1:100) from ECD/Her2-immunized mice was able to reduce the proliferation rate of SK-BR-3 cells by 13% \((p<0.05)\), while such an effect could not be detected with the diluted sera from PBS-immunized mice. Given that antibodies against the Her2/neu receptor do exist in sera from ECD/Her2-immunized mice, and not in sera from PBS-immunized mice (Figure 3), it is clear that these antibodies have significant anti-proliferative effects in SK-BR-3 cells.
Response to Reviewer 2: Eric von Hofe:

1. “When Balb/c and HHD” – awkward, suggest rewording

This phrase was replaced by “When immunized Balb/c and HHD” as suggested.

2. Indicate in the Conclusion of the Abstract that specific cellular and humoral responses were observed as well as efficacy.

The sentence “Specific cellular and humoral responses were observed as well as efficacy” was added as suggested.

3. “is also observed in various human cancers” – awkward, suggest rewording

This phrase was replaced by “is also observed in various other human cancers” as suggested.

4. “in tumor prevention” – awkward, suggest rewording

This phrase was replaced by “to reduce tumor growth” as suggested.

5. “In the Results section, the first paragraph seems to repeat some of the information already presented in the Methods section.”

We have erased some information as suggested.

6. “It would be very helpful if the numbers of mice in all the experiments was given.”

The numbers of mice used in all experiments is given in page 8 “Groups of 9 mice” and in page 10 “with ECD/Her2 (n=4) or PBS (n=4)”.

7. “SK-BR-3 cells are used to check for antibodies, though D2F2/E2 or wild-type D2F2 are used for in vivo study – why?”

We used transplantable tumor models for assessing the capacity of ECD/Her2 to elicit antitumor responses in mice. Therefore, we used D2F2/E2 tumor cells for inducing tumors in BALB/c mice and ALC.A2.1.hHer2 tumor cells in HHD mice. The SK-BR-3 control cell line was used for checking the activity of collected sera in vitro because this cell line overexpresses HER-2/neu as a result of gene amplification and therefore should be sensitive to the action of anti-HER-2 antibodies, in a manner analogous to that induced by Herceptin.

8. “Similar to 7., it would be of interest to see the activity of CD8+ T cells from immunized mice against wild type D2F2 cells.”

We have observed negligible levels of cytotoxicity against wild type D2F2 cells and therefore we did not include these negative data in Fig.6. However, we now mention this in “Results”.
9. “Similar to 7. and 8., it would be good to know something about the growth of non-transfected ALC cells in immunized HHD mice.”

In our recently published works, we have shown that wild type ALC cells grow rapidly in HHD animals immunized with immunogenic epitopes of the extracellular domain of HER2 (Gritzapis AD et al., Cancer Res 2006, 66(10):5452-5460, Gritzapis AD et al., J. Immunol. 2008, 181(1):146-154). Therefore, given the lack of sufficient numbers of HHD mice in our animal facility, we decided not to include this control in our in vivo studies.

10. “Given the different cell types used, it is not entirely clear why the authors focus on the importance of CTL activation at the end of the discussion. A few more words of explanation might be helpful.”

Given that novel approaches in the field of peptide-based vaccines include polypeptides or long-peptides encompassing a variety of immunogenic epitopes, our intention was to show that ECD/Her2 contains multiple immunogenic epitopes restricted by various alleles. As a first step in this direction, we showed in the present manuscript, by using 2 different mouse/tumor models, that there are at least 3 different alleles restricting immunogenic epitopes of ECD/Her2, namely HLA-A2.1, H-2Kd and H-2Dd. Work is now in progress in our laboratory to identify those epitopes.