Author's response to reviews

Title: Human papillomavirus (HPV) type 16 E7 protein bodies cause tumour regression in mice

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Author's response to reviews: see over
Re: Revisions to Human papillomavirus (HPV) type 16 E7 protein bodies cause tumour regression in mice

We did all the revisions as requested by the reviewer and we also addressed all the comments. Please find our responses to the reviewer in red below.

Yours sincerely
Inga Hitzeroth

Reviewer 1

Major Compulsory revisions
1. The reason that insect cells and baclovirus are used to isolate Zera protein bodies, if one of the proposed advantages of using Zera is to express recombinant protein in whole plants, should be clarified.

The reason for using baculovirus and insect cell-produced protein bodies has been clarified and justified under section 3.4 Determination of the role of Zera® protein in the immune response. The following was added: “As we were not able to express 16E7SH alone in plants to any reasonable concentration for inoculation doses (3.1), we expressed this recombinant protein in *E. coli* as this method resulted in the production of adequate amounts of 16E7SH easily. In addition, the Zera® PBs used in these experiments were produced in insect-cells as their expression using this method is high (+/- 40 mg/L), and recovery is very efficient. In addition, Zera® PBs from insect cells and tobacco plants are very similar in that they are round, range in size from 0.5 to 1 µm, membrane-surrounded and do not undergo post-translational modification [32].”

We have also made it more clear that we used insect cell produced PB in the final paragraph of the introduction.

2. eGFP has been reported to be immunogenic in mice. The rationale for including this as a control in these experiments should be clarified. (Figure 3-4)

The report of eGFP immunogenicity in mice has been referenced and the use of this control has been discussed in the discussion section of the paper. The following paragraph was added: “The ability of the plant-produced ZERA-16E7SH PBs to cause tumour regression
was shown to be significant and similar to that of the DNA vaccine equivalent. Further co-inoculation of ZERA-16E7SH PBs with adjuvant, however, did not significantly enhance tumour regression suggesting that Zera® has an adjuvanting effect by itself. Inoculation of tumourigenic mice with plant-produced ZERA-GFP lacking 16E7SH also did not result in tumour regression suggesting that Zera is not immunogenic. Despite the fact that it has been shown that enhanced GFP is minimally immunogenic in C57/BL6 mice [34], the lack of tumour regression observed when mice were inoculated with ZERA-GFP further supports the evidence that the 16E7SH is the immunogen causing tumour regression and that Zera® does not contribute to this.

3. The reason tumors were run for 5-6 weeks in Figure 5, but only 2 weeks in Figure 3 should be explained further. The way these experiments are reported raises the question whether differences not seen in some of the groups in Figure 3 would have become evident if tumors were propagated longer.

We agree that it would be helpful to present different tumour experiments with similar/same observations period. Unfortunately, some tumours became bloody in animals of the control group (empty vectors) and accordingly to the german animal testing regulations it was necessary to terminate the experiment at day 14. This was addressed in the Method section 2.11 Tumour regression experiments „Mice were sacrificed when the tumour size reached 400 mm² or when tumours were bleeding.”

The presentation of a single timepoint and 2 identical experiments (Figure 5) is less informative than the plots over time seen in Figure 3. We only measured the tumour size at the beginning and end of the experiments and therefore no tumour sizes are available for time points in between. We also wanted to demonstrate that this was not a once off result, but that we got very similar results in a second experiment where the tumour sizes were similar and that is why we presented two experiments to demonstrate this.

Minor Essential Revisions

1. Figures (3-7) and/or figure legends should show p values and clarify which timepoints or comparisons are statistically significant.

We added the p values in figure legends in Figures 4, 5, 6 and 7.

2. Nomenclature of vectors should be made consistent between text and figures.

Nomenclature of both the eGFP (GFP was changed to eGFP) and pTH (pTHamp in text changed to pTH) vectors has been made consistent between the text and figures. ‘ Adj’ in figure 3 has been changed to ‘IFA’ to keep consistency throughout all the figures. GFP was changed to eGFP in text and Figures 3 and 4 to make it consistent.

Discretionary revisions

1. This manuscript could be improved if it were more focused on the role of Zera as a novel vaccine adjuvant rather than use as potential therapeutic cervical cancer vaccine, which is what the introduction currently prepares the reader for.

The aims of the paper have been clarified in the final paragraph of the Introduction. The last paragraph in the introduction was changed to: "We explored the development of a plant-produced, potentially therapeutic protein-based vaccine that could cause regression of HPV lesions in humans infected with HPV-16, and which would also be affordable in developing countries. We investigated whether HPV-16E7SH and Zera® protein bodies can induce tumour regression in mice. This was tested either by administering 16E7SH-Zera® fusion proteins or by administering a mixture of Zera® protein bodies (PBs) and 16E7SH protein to tumourigenic mice. The proteins were produced in three different expression systems: HPV-16E7SH protein fused to Zera® was expressed in plants, HPV-16E7SH was produced in E.
coli and Zera® PBs were produced in insect cells. Immune responses of the plant-produced protein were compared to those of the well-characterised E7SH DNA vaccine in the murine model; tumour regression as well as cell-mediated and humoral responses were analysed. Finally, the adjuvanting properties of the Zera® protein were investigated.”

We also removed “plant produced” in the sentence below in the abstract.

In this paper, we characterize the immunogenicity of a (plant-produced) therapeutic vaccine that targets the E7 protein of the most prevalent high-risk HPV - type 16 – the gene which has previously been shown to be effective in DNA vaccine trials in mice.

2. Although immunogenicity and tumor control are seen when mice are vaccinated with a shuffled HPV 16 E7 sequence, the presence of E749-57 in the vaccine as a preferred H2Db restricted epitope is likely responsible most of tumor control and ELISPOT and Granzyme B results. How inclusion of this epitope in the vaccine this limits translation of these findings clinically to humans with outbred MHC class I alleles may be discussed.

The following was added to the discussion: “For safety reasons the use of wildtype HPV-16 E7 for vaccination is not feasible in humans. Approaches like the introduction of point mutations into the E7WT gene, however, lead to an unwanted loss of naturally occurring epitopes that is potentially associated with a decrease in vaccine efficacy. We used a rearranged (“shuffled”) E7 sequence which lacks transforming properties [11]. Ultimately this non-transforming HPV-16E7SH supplies all potential naturally-occurring T cell epitopes, covering the broad range of MHC restriction. Consequently, prior knowledge of the patient’s HLA-haplotype is not required which is especially important in the outbred human population. In addition, a more potent immune response may be induced, involving all occurring HLA-restriction elements in the vaccine.”

3. A rationale proposed for these experiments centered on the use of Zera sequences and plant expression to allow high-level and inexpensive production of a therapeutic protein based vaccine targeting HPV 16 E7. However, data presented from these experiments suggest that DNA based vaccination produces superior tumor control (Figure 5) and comparable immunogenicity (Figure 6). The discussion could explore the ramifications of this in more depth.

We feel that the DNA vaccine has been discussed in depth in the discussion and adding more might make it too repetitive. The following paragraph is in the discussion and the role of ZERA in the DNA vaccine has also been discussed in a further paragraph.

“In general, the DNA-based vaccines were more efficacious than the protein-based vaccines in the context of control of tumour growth (Fig. 5), which probably reflects in part the fact that DNA vaccines induce more Th1 than Th2 responses after intramuscular injections. The pTH-16E7SH construct did, however, also induce a moderate IgG response, as measured by ELISA (Fig. 7). Additionally, IFN-γ levels of splenocytes isolated from mice inoculated with pTH-ZERA-E7SH were elevated compared to mice inoculated with pTH-16E7SH (Fig. 6). This trend was also observed in Granzyme B ELISPOT assays as well as in chromium release assays.”

**Additional changes that were made:**

Figure 3 was changed so that A and B have same scale on X-axis.

In results 3.3 was missing so we relabelled all sections.

Sentence was added in introduction second last paragraph, last sentence: “A further advantage of such protein bodies is that their co-administration with recombinant vaccines may have an adjuvant effect and enhance the immune response as a result of their particulate nature.”
Methods Section 2.6 was moved up to be 2.5 and all other sections relabelled

The following sentence was added to 2.7 Expression of 16E7SH in E. coli

“16E7SH protein for animal trials was expressed in *E. coli* as detectable levels of its expression in plants was never achieved.”

2.8 Insect cell culture and baculovirus production of recombinant proteins was changed to Insect cell culture and baculovirus production of protein bodies.

Heading 2.9 was removed and the whole paragraph shortened to “Zera® protein bodies were isolated from frozen *Sf*9 cell biomass previously infected with the selected baculovirus. Zera® PBs were recovered as described by Torrent et al. [26]. PBs washed with LPS-free water were characterized by SDS-PAGE and confocal and scanning electron microscopy.”

Results 3rd paragraph a sentence was added here “For further purification studies and preparation of recombinant proteins for animal experiments, infiltrated plants were harvested at 7 dpi, as this was the time at which the highest protein levels were visualised.”

The following sentence was deleted from end of paragraph 4 in results.

In contrast, 16E7SH protein alone, (expected size of 18 kDa), was not detected throughout the same time trials, indicating the positive…”