Author’s response to reviews

Title: Nasal double DNA adjuvant induces salivary FimA-specific secretory IgA antibodies in young and aging mice and blocks Porphyromonas gingivalis binding to a salivary protein

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Author’s response to reviews:

Dr. Liam Messin
Editor
BMC Oral Health

Re: Manuscript OHEA-D-19-00113

Manuscript “Nasal double DNA adjuvant induces salivary FimA-specific secretory IgA antibodies in young and aging mice for blocking Porphyromonas gingivalis binding to a salivary protein”

Dear Dr. Messin:

Thank you for your letter dated June 17, 2019 regarding our original manuscript entitled: “Nasal double DNA adjuvant induces salivary FimA-specific secretory IgA antibodies in young and aging mice for blocking Porphyromonas gingivalis binding to a salivary protein”. We are pleased to learn that the external reviewer considers our study of interest and have raised helpful
comments. We have incorporated the reviewer’s critiques into our revised manuscript (highlighted in red).

We feel that these critiques were very helpful and they have improved our manuscript. Our point-by-point responses to the reviewer’s comments are provided below.

We take this opportunity to express our gratitude to the editor and the reviewer for their constructive and useful remarks. We also thank you for allowing us to resubmit the manuscript.

We hope that the revised manuscript is now acceptable for publication in BMC Oral Health ASAP.

Point-by-point responses

Editor Comments: The authors should address the reviewer’s concerns and provide a clarity to the materials and methods section. The extensive English language editing is required for clarity.

Authors’ response: We have carefully examined the reviewer’s comments and our point-by-point response including clarification of the Material and Methods section, can be found below. The manuscript was edited by the native English speaker (Forte Science Communications; Forte, Inc., Tokyo, Japan) in order to provide a clarity of the text.

Reviewer reports:

Lesley Ann Bergmeier, PhD (Reviewer 1):

General comments.

Reviewer’s comment 1: The authors are using a gst-fusion protein of rFimA produced in E. coli, but it was not clear if this is the full-length protein or a fragment. Fig 1 suggests it is 41kDa but there is no indication if this is the fusion protein. Gst has a molecular weight of about 26kDa so that would mean a fragment size of about 15kDa. I was confused as the papers to which they refer (refs 25,26) suggest that protein has a molecular weight of between 37-38 with variants at 43-48kDa. While Cai et al 2019 (ref 12) show a band at about 60kDa. This would mean that the rFimA-gst fusion protein should have a mol wt of at least 60kDa. Why did the authors not use anti gst antibodies to determine the mol wt of the fusion protein and to look for any breakdown products? It is not until the results section that it becomes clear that the gst has been cleaved from the fusion protein.

Authors’ response: We apologize for the confusion created by our description in the original manuscript. We have provided the detailed method for the purification of rFimA in the Material
and Methods section of revised manuscript. In brief, the supernatants from ultrasonication of E.
coli BL21 transformants carrying PYT1245 plasmid were applied to GST-binding affinity
column. The rFimA protein (MW; approximately 41 kD) was eluted out by cutting GST-rFimA
fusion protein off using PreScission proteaseTM. Based upon Western blot analysis (Fig. 1c), our
preparation of rFimA do not contain any GST protein, which showing the size of approximately
41 kD.

Reviewer’s comment 2: The Authors use ELISA to detect IgG and IgA antibodies and seem to
assume that the latter are SIgA but do not give evidence of secretory component. As far as the
ELISPOT is concerned direct secretion from the lymph node derived B cells might be expected
to show dimeric IgA but since the antibodies acquire the secretory component prior to passage
though the epithelium, the authors might be more cautious in the assumption of SIgA in saliva-
without a demonstration of SC.

Authors’ response: The reviewer’s point is well taken. As this reviewer mentioned above,
salivary IgA Abs are secreted by acinar cells of the salivary grands. Thus, detection of IgA Abs
in saliva by using ELISA has been accepted as the assessment of SIgA Ab responses (ref. No.
30; Fukuyama Y. et al.).

Reviewer’s comment 3: There is evidence in the literature that antibodies to a variety of P.ging
proteins can be induced in both IgG and IgA compartments and their function can be
contradictory in terms of protection or enhancement of progressive periodontal disease. Some
of the data presented suggest that that IgG antibodies might interfere with the ability of IgA to
inhibit adhesion to Statherin- but this is not very well explored, and the conclusions are
confusing.

Authors’ response: We appreciate the comments raised by this reviewer. In order to clarify the
roles of rFimA-specific IgG Abs for the prevention of Pg binding to Statherin, whole saliva from
mice given rFimA plus DA was employed for the Pg-binding assay. Our results showed that
whole saliva revealed essentially the same levels of inhibitory activity as IgA enriched saliva
from mice immunized nasally with rFimA plus DA (revised new Figure 5). These results show
that FimA-specific IgG Abs in saliva do not play key a role in the prevention of Pg binding to
Statherin. We have added these findings in the Results section of the revised manuscript (page
13, lines 2-6).
Specific comments.

1) Abstract: Page 2: Line 16: This line does not make sense. Should perhaps read "which were preincubated with saliva from which IgG antibodies had been removed using protein G column"

Authors’ response: We appreciate this reviewer’s kind suggestion. We have changed the sentence as indicated above (page 3, lines 13-16).

2) Background: Good description of the literature but the paper would benefit from comments on what is known about IgG/IgA antibodies in perio.

Authors’ response: We have added a couple of sentences describing roles of IgA and IgG Abs in periodontal disease (page 6, lines 11-20, the Background section of revised manuscript).

3) Materials and methods: Page 6 line 9. The authors mention that the fusion protein is purified on a Glutathione Sepharose 4B gel- implying that it is a full-length fusion protein. Gst+ rFimA should be>60kDa but Fig 1b and 1c has the fusion protein at 41kDa- this needs to be explained/clarified.

Authors’ response: We apologize for the confusion created by our description in the original manuscript. As we described in Authors’ response to General comment section (Reviewer’s comment 1), the purified protein contains rFimA only without any GST protein.

4) Materials and methods: Page 7, line 7: Do the authors have data on the induction of DCs with DA alone in this series of experiments. This would appear to be a control that would be important for the cellular aspects of the study.

Authors’ response: Unfortunately, we do not have data for the induction of dendritic cells (DCs) with double adjuvant (DA, pFL plus CpG ODN) alone. However, our results showed that numbers of DCs induced by rFimA alone are essentially the same as those seen in the naïve mice (1.8 %, average DCs percentage in naïve mice). In contrast, rFimA plus DA resulted in significantly increased numbers of activated DCs. Further, it has been shown that pFL and CpG ODN specifically target DCs for their proliferation and activation (ref. No. 22; Fukuiwa T. et al., ref. No. 30; Fukuyama Y. et al.). In this regard, DA alone most likely induces the activation and proliferation of DCs.
5) Materials and methods: Page 7 line 20: The color reaction--- What substrate was used? OD415 - this seems an odd OD for an HRP substrate. More detail is required here. ELISPOT analysis: Have the authors looked at Total AFCs in the lymph nodes? Why was anti-gst not used to look at the immunogenicity of the fusion protein. Not enough detail.

Authors’ response: We have added the details of ELISA in the Materials and Methods section of revised manuscript. As we mentioned above, since we used purified FimA but not GST-FimA fusion protein, we did not assess anti-GST immune responses including the numbers of GST-specific antibody forming cells (AFCs). Since the numbers of total AFCs in the various mucosal tissues including submandibular glands have been previously reported elsewhere (ref. No. 32; Mega J. et al), we did not assess them in the present study.

6) Materials and methods: FACS analysis: I would like to see an example FACS plot. What gating strategy was used? It could be given as a supplementary Fig.

Authors’ response: We have provided the typical FACS plot and gating strategy of flow cytometric analysis in the supplementary information (Supplementary Figure 1 in the revised manuscript).

7) Materials and methods: Blocking assay, This is a crucial functional assay for this study but is very poorly described. It is not at all clear what the readout for the assay is. ATP in live bacteria? It would be impossible for anyone to use this assay as there is not enough detail of how it was done.

Materials and methods: Page 9 line 6: What is the read out for the assay.

Authors’ response: We appreciate this reviewer’s constructive comment. We have provided additional details of blocking assay for P. gingivalis binding to salivary statherin in the Materials and Methods section of revised manuscript (page 10, lines 9-26). In brief, the numbers of P. gingivalis was assessed by The BacTiter-Glo™ Microbial Cell Viability Assay. This commercially available assay kit provides a method for determining the number of viable microbial cells in culture based upon quantitation of the ATP present. The homogeneous assay involves adding a single reagent (BacTiter-Glo™ Reagent) directly to bacterial cells cultured in medium and measuring luminescence.
8) Results: Page 9 line 20- the use of "Prescission (Precision) Protease to purify the rFimA is not mentioned in the M&M. This part of the methodology should be in M&M. It is crucial to the clarity of the paper in terms of the purity of the rFimA.

Authors’ response: We totally agreed with this reviewer’s comment. We moved “Purification of rFimA protein” section to Materials and Methods section of revised manuscript.

9) Results: Page 11 line 7: What tissues are included in this - Waldeyers ring? Tonsils?

Authors’ response: In mice, NALT can be seen in cross-section as paired lymphoid tissues on either side of the nasal septum at the base of the nasal passages, directory dorsal to the soft palette of the oral cavity (Asanuma, H., et al, J. Immunol. Methods, 202: 123-131, 1997). NALT is represented in humans by Waldeyer’s ring of tonsils and adenoids.

10) Results: Page 11 line 23. This is difficult to follow as the assay and the readout are not clear.

Authors’ response: We have rewritten the results of binding assay section. Since the details of assay including the read-out were provided in the Materials and Methods section, we focused to describe the levels of ATP activity and their relations with the number of P. gingivalis in the Results section of revised manuscript (page 12, line 24-Page 13, line 6).

11) Discussion: Page 12 line 24- SLGs have higher IgA AFCs than SMGs. Page 12 line 26-contradiction? SMGs are the major mucosal effector tissues in the oral cavity. This is a very good point that this study indicates that SMG and SLG are both part of the effector sites- this would be supported by SLIT therapy studies where IgA is induced in SLGs.

Authors’ response: The reviewer’s point is well taken. We modified the sentences indicated above as follows: “Based upon these finding, SLGs should be named as mucosal effector tissues in addition to SMGs which are known as one of the major mucosal effector tissues for the production of IgA Abs in the oral cavity” (page 14, lines 8-15).

12) Conclusion: Brief and to the point.

Authors’ response: We appreciate the reviewer’s positive input.