Author’s response to reviews

Title: Salivary pellets induce a pro-inflammatory response involving the TLR4 - NF-κB pathway in gingival fibroblasts

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Version: 1 Date: 12 Feb 2016

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Author’s response to the comments

Reviewer #1 comments:

1. In "Materials and methods", the human saliva was collected from the group of authors, which means saliva from several donors. So does all the data in study come from one specific donor or different donors?

Response to reviewer’s comment 1:

The saliva used in the manuscript was collected exclusively by the authors. Data represent the data of three donors. Moreover, according to previous studies from Cvikl et. al. 2015 the differences among the saliva donors in chemokine expression is negligible. We added that information to the manuscript.
2. Page 2, line 34, and page 7, line 31. failed to find any data about chemokine expression in response to BAY11-7082 in this manuscript.

Response to reviewer’s comment 2:

In our study BAY11-7082, a selective and irreversible inhibitor of NF-κB activation, significantly reduced inflammatory impact of saliva and salivary pellet. Thus, previous studies and our data suggest that BAY11-7082 does not exert inflammatory response of primary gingival cells. Usually it requires activation of NF-κB to use the inhibitor according to his purpose. The basal NF-κB activity is usually very low and therefore the application of BAY11-7082 not recommended.

3. What's the LPS concentration in a series of dilutions of saliva pellet?

Response to reviewer’s comment 3:

One of the major findings of your study is that the inflammatory response of primary gingival cells to human salivary pellet is caused by LPS. Therefore the investigation of the LPS concentration of salivary pellet revealed a LPS concentration of 1900 Units/ml. Thus we did not measure in advance the LPS concentration of the diluted salivary pellet preparation. However, the LPS induced chemokine expression in gingival cells revealed a robust inflammatory impact even after a series of washing steps.

4. How is the TLR4 expression in whole saliva and saliva pellet?

Response to reviewer’s comment 4:

The results of our experiments show that Toll-like receptor 4 (TLR4) is a main component of inflammatory signal transduction induced by LPS loaded salivary pellet. TLR4 is an evolutionary conserved element of the innate immunity and therefore it can be found on the outer cell membrane of various human cells; TLR4 is moreover restricted to cellular components.
Therefore saliva and salivary pellet do not contain TLR4. Thus also here TLR4 expression is not in the focus of this research.

5. Page 6, line 61. Did authors try other MyD88 inhibitor? How's the MyD88 expression in response to pellet after blocking TLR4 by TAK-242?

Response to reviewer’s comment 5:

This was not investigated in the present study. We used TAK-242, a TLR4 inhibitor, instead of MyD88 inhibitor. MyD88 is a downstream mediator of TLR4 signaling and the typically used MyD88 inhibitors are not as potent as TLR4 inhibitor. In addition, in our study we wanted to inhibit TLR4. MyD88 therefore is not a specific inhibitor for TLR4 alone.

6. Move Table 2 and Table 3 to "supplementary data".

Response to reviewer’s comment 6:

We thank the reviewer for the suggestions and moved table 2 and table 3 to supplementary data.

7. Table 4. Any insight about elevated expression of CXCL 8 in response to autoclaved pellet?

Response to reviewer’s comment 7:

This is a surprising finding since autoclaved saliva causes a dramatic decrease of inflammatory activity of the salivary pellet. These data tell us that we have to interpret the finding carefully with regard to the presence of endotoxins, which are considered to be stable at autoclaving. Support for this comes from our experiments with isolated LPS (Suppl. Table 2). We have reformulated the overall conclusions now being more careful with regard to the ligand(s) provoking the pro-inflammatory answer of the gingival fibroblasts.
8. Table 5. Add "unit of measurement".

Response to reviewer’s comment 8:
Table 5 shows the x-fold changes of gene expression normalized to the unstimulated control. We changed the table caption accordingly.

9. Figure 6, how the chemokine expression and p-P65 in pellet without LPS when exposed to TAK-242.

Response to reviewer’s comment 9:
In Figure 1 we show the impact of salivary pellet on the chemokine expression of gingival fibroblasts relative to the unstimulated and therefore untreated control. The relative chemokine increase of gingival fibroblasts stimulated with 1-, 2-, 4- times washed pellet is over 1000-fold. This relative and strong increase is expressed as x-fold change. So even the washed salivary pellet elevated chemokine gene expression in gingival fibroblasts. In our experiments we tried to remove LPS from the dense salivary pellet, but our method was not adequate to filter LPS from the pellet because of the pellet density. However, 4-times washed pellet was not so dense and we are able to show in Figure 6 the chemokine expression after removal of LPS. Moreover, in experiments TAK-242 at 25 µM alone, the chemokine expression was unchanged to untreated control, therefore TAK-242 at 25 µM does not elevate chemokine expression in gingival fibroblasts (data not shown). We addressed this in the manuscript.

10. Figure 6B, change "p-NF-KB" to "p-P65".

Response to reviewer’s comment 10:
We thankfully follow the suggestion of the reviewer and adopted the figure accordingly, changing "p-NF-KB" to "p-P65" in Figure 6B.
Reviewer #2 comments:

1. The only real drawback to the manuscript is the slight reformulation of certain sentences to improve the English language.

Response to reviewer’s comment 1:
The manuscript underwent a critical proof-reading as suggested.

2. The authors also may wish to consider changing 'gingiva' fibroblasts to 'gingival' fibroblasts.

Response to reviewer’s comment 2:
The reviewer recommended to change 'gingiva' fibroblasts to 'gingival' fibroblasts. We followed the suggestion and changed this word throughout the manuscript.

3. Please also consider changing 'saliva pellet' to 'salivary pellet'.

Response to reviewer’s comment 3:
The authors thank the reviewer for the advice and changed the wording from 'saliva pellet' to 'salivary pellet' throughout the manuscript.