Author's response to reviews

Title: The effect of Lipoxin A4 on the interaction between macrophage and osteoblast: possible role in the treatment of aseptic loosening

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Version: 2 Date: 10 April 2009

Author's response to reviews: see over
Dear Editor Mark Todd,

I am so glad to receive your email and the comments enclosed. According to the reviewers’ report, the manuscript has been carefully revised. The following are the point-by-point responses to these concerns. All the sentences supplemented or revised are marked red in revised manuscript.

**Part A: Editorial requests**

1. Please state the name of the ethical committee / board, which granted you permission to perform this investigation, in the Methods section of your investigation.
   Reply: This state has been put into the *Methods section* of the revised manuscript.

2. Please revise your Authors' Contributions section, so that it adheres to these guidelines: http://www.biomedcentral.com/bmcmusculoskeletdisord/ifora/#authorscon
   Reply: This section has been revised according to above guidelines.

**Part B: Comments from the first reviewer**

1. I would suggest that in title “inflammation resolution” could be omitted.
   Reply: These two words had been deleted in the title. Now, we also feel that the title can describe the content of the paper more accurately than the original one.

2. The authors should indicate the commercial source of the LXA₄ they use in the study.
   Reply: Sorry about this mistake. It was added in the *Methods Section*.

3. In the first set of experiment the Authors use different concentrations of PMMA (from 0.1 mg/ml to 1 mg/ml). The increase in pro-inflammatory cytokine production is statistically significant at all the concentrations tested. In the following experiments, the Authors use 1 mg/ml of PMMA in the presence of LXA₄. The Authors should discuss why they use this concentration in the following experiments, considering that probably the effect reaches the highest plateau, rendering more difficult to evaluate an effect of LXA₄. However, even in these experimental conditions, LXA inhibits cytokine production in a range of concentrations between 10 nM and 100 nM. On the other end, the endogenous levels of LXA measured in the supernatants are about 2 nM. This concentration should be used also in the experiment when exogenous LXA is tested and probably PMMA should be used at a lower concentration.
   Reply: Thank you so much for your suggestion. The results and related discussion of 1 nM LXA₄ was added to revised manuscript.

   Firstly, in most of the papers studying on LXA₄, the concentration of exogenous LXA₄ ranges from 1 to 100 nM *in vitro*. So, in our study, we also chose 1, 10, 50 and 100 nM as 4 different concentrations. But, as a result, there was nearly no difference between the effect of 1 nM and 10 nM LXA₄. That is why we just reported the data of 10, 50 and 100 nM LXA₄ in the original manuscript.

   But, it is true as the reviewer pointed out, to the readers of this paper, this spectrum of concentration in the manuscript is not complete, because it did not include the concentration...
of endogenous LXA₄ we measured in our model. So, we added the original data of 1 nM LXA₄, as shown in revised Figure 2 and 3.

We also added some related explain in the Discussion Section.

Secondly, as for the concentration of PMMA, in our preliminary experiments, we tried different final doses, from 0.1 mg/ml to 1 mg/ml, even higher. And it was shown that PMMA higher than 1 mg/ml concentration could trigger more serious inflammatory response in cultured macrophages (data not shown in the original manuscript). So, that means the highest dose we used in our study did not reach the plateau.

As it was described in the Methods Section, PMMA was first weight and sterilized in 70% ethanol before it was diluted in PBS as storing solution at 5mg/ml. Then this solution was added into cell culture medium at the final doses indicated. We have two methods to increase the final PMMA dose. First option is increasing the volume rate of storing solution to culture medium but this will dilute culture medium and is not good for cell growth. Second option is increasing the concentration of storing solution, but, 10 mg PMMA could not be well distributed in 1 ml PBS. They will agglomerate. That means we can not tell exact dose of it, maybe 7.5 mg/ml, maybe 8.5 mg/ml or higher.

So, as our experience, PMMA higher than 1 mg/ml could trigger more serious inflammation but we just use 0.1 to 1 mg/ml PMMA to stimulate the cells.

We added related explain in the Results Section in revised manuscript.

4. The experiments carried out to evaluate the effect of the inhibition of 15-LO should be better described. It is not clear which cell type was target by the siRNA.
Reply: It has been added into Gene Silencing Part of Methods Section.

5. Following the results of these experiments, The Authors suggest that LXA4 is indeed the mediator responsible of the effects described in this paper. However the formation of other mediators could be inhibited by the 15-LO siRNA. The Authors should discuss this possibility.
Reply: It is a good suggestion. It was added to the last paragraph of Discussion Section.

6. It could be interesting to define the exact role of LXA, carrying out experiments in which an inhibitor of LX receptor (i.e. Boc compound) is used. This experiment will unequivocally demonstrate the direct involvement of LXA4.
Reply: That is true. It will be more convincing if we can get the similar results when we inhibit or block some other points of whole signal pathway of LXA₄, including its receptor.

But, as we know, the receptor of LXs was called as ALX. It belongs to the family of chemotactic receptors and clusters with formyl peptide receptors. Therefore, it was also denominated formyl peptide receptor like 1 (FPRL1). Now it was confirmed that this receptor recognizes a variety of peptides, synthetic, endogenously generated, or disease associated, including LXA₄. That means blockage of this receptor will cause some unwanted, unspecific effects. For this reason, most of the papers studying on the effect of LXs use exogenous LXA₄ or siRNA of 5-LO or 15-LO.

Part C: Comments from the second reviewer
1. Looking at the results of figure 5, what the authors think about of the levels of cytokine do not have reduced in macrophage co-cultured with OB and treated with PMMA when compared with macrophages stimulated only with PMMA? In this system there was lipoxin production. The authors think that LXA4 added exogenously has the synergistic effect with that produced endogenously?

Reply: It is a good question.

First it was confirmed that, in the peri-implant tissue of patients with aseptic loosening and animal models, macrophage is a main cell type to the beginning and developing of inflammation. But, OB is not just responsible for bone formation. Now, it is believed that OB, especially the interaction between OB and macrophages, is necessary to the amplification of inflammation and migration of osteoclast to peri-implant tissue (seen in Introduction Section of manuscript).

That means in our co-culture system exposed to PMMA, like the reviewer said, endogenous Lipoxin will produce. While under this condition, inflammation will be more serious than macrophages cultured alone. We can not completely separate these 2 opposite effects on the cytokine production. But, one thing we are sure is that after Lipoxin production was blocked by siRNA for 15-LO, inflammatory response will be more obvious (Seen in Figure 5 and 6). We think this result could support our conclusion.

That is also the reason we feel quite interested in the effect of Lipoxin and choose the co-culture system to evaluate it. This part of discussion already existed in the last but one paragraph of Discussion Section.

2. What explain the increase of levels of GM-CSF when the 15-LO is inhibited compared with culture of macrophage and PMMA only?

Reply: We think that GM-CSF is just like other inflammatory cytokine we measured in the study. In local inflammatory site, on the one hand, they can trigger the production of Lipoxin, on the other hand, as a feedback, Lipoxin will inhibit the stimulation which induced those inflammatory cytokines. So, when the 15-LO is inhibited, GM-CSF is increased.

3. What explain the fact of do not to have diminution of bone resorption in system contained macrophage + OB + PMMA-figure 6? In this system there is lipoxin production?

Reply: Please see the reply to question 1.

4. In the last paragraph of discussion the authors cite that they used PMMA particle to mimic the inflammation in AL patients. This affirmation is not correct.

Reply: This sentence has been revised as seen in the last paragraph of Discussion Section.

5. Why the authors conclude that LXA4 has a favorable inhibitory effect on PMMA-induced inflammation in co-culture system? What means favorable in this in vitro system?

Reply: The word “favorable” has been deleted in revised manuscript.

When we used word “favorable” in the original manuscript, we meant that LXA4 could inhibit PMMA-induced inflammation since this was the main reason to cause AL in clinical. But, it is right, as the reviewer pointed, that we just did in vitro experiments. It was not suitable to describe this way. So, this word was deleted.
6. In figure 1, is better to use “time after PMMA stimulation” and not “PMMA administration”. The word “administration” gives idea of in vivo studies.
Reply: It has been revised.

7. The Figure 7 should be advanced to Figure 4, because its results proves that the use of siRNA for 15-lyoxigenase works.
Reply: The figures, including their illustration, and related explain in Result Section had been revised.

8. The correct way to synthesize the name lipoxin A4 is LXA4 and not LAX4. Please change all those mistakes: page 7, 1º line, 9º line, 17º line.
Reply: Above mistakes had been revised.
We also found that the abbreviation of Lipoxin A4 in most of the papers published was LXA4, while we used LXA4 in the original manuscript, so, we revised all the LXA4 to LXA4.

9. Page 8 line 9: to cite number of figure
Reply: Added in the revised manuscript.

10. In Figure 2, the dose of LXA4 used is represented by nm. What is it means? If it represents nanomolar, the correct way to write it is nM.
Reply: Sorry for the mistake. It has been corrected. Since the figures were uploaded as JPEG file, we did not mark this change in red.
We also corrected the same mistake appeared in Figure 3 and some other places in the manuscript.

11. In Figure 5, I’d suggested the authors to plot the graphs at different way. Or put all the parameters analyzed in the same graph, or divide in four panels, each one for each parameter.
Reply: It has been changed to for panels, each one for each parameter.

12. In page 10, 3º paragraph, 4th line of the discussion, please change the word excrete to secrete.
Reply: It has been revised.

Sincerely,

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