Author's response to reviews

Title: Effects of antibody to receptor activator of nuclear factor kappa-B ligand on inflammation and cartilage degradation in collagen antibody-induced arthritis in mice

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Version: 4 Date: 20 November 2014

Author's response to reviews:

Response to Reviewer 1:

Thank you very much for reviewing our manuscript. Your comments and our responses to them are as follows.

1- The increased bone mass, around 3% depicted on the figure 2 is quite surprising high given the fact that the treatment with anti-Rankl antibody is short (9 days). Is it realistic? Could the authors provide a clear histomorphometric analysis of the data?

Response:

Thank you for your question about the activity of the anti-RANKL antibody used in our study. It is one of the most important points of our study to use anti-RANKL antibody that is effective in vivo. Fortunately, it was previously reported that OYC1 anti-RANKL antibody could increase bone mass in mice (reference number 13). They used the antibody at the dose of 5 mg/kg body weight, we decided to use the same amount of OYC1 in our study. Hence it might be said that increased bone volume (BV/TV) in our model was the result that we
expected. According to your suggestion, we revised Figure 2 by incorporating the results obtained in RA+ mice. In addition to BV/TV, trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular space (Tb.Sp) are shown. Increase in BV/TV was observed again in RA+ mice (revised Figure 2C). On the contrary, the effects of OYC1 on Tb.Th (RA- and RA+ mice), Tb.N (RA- mice), and Tb.Sp (RA- and RA+ mice) were insignificant (revised Figure 2D-F, H, I). We speculate that these results were because the systemic bone metabolism was possibly nearly normal with low level of bone resorption even in the RA+ mice, the effect of OYC1 on bone metabolism in the metaphysis of tibias was small.

2- A quantitative analysis of serum levels for markers of bone remodeling would be helpful.

Response:

Thank you for your suggestion. We tried to quantify NTx (amino-terminal type I collagen crosslinks), a marker of bone resorption, in all the mice that we used in this study. Unfortunately, NTx values in the sera were all under the detection level (<40.0 nmol bone collagen equivalent/L) irrespective of the treatments. Therefore there is a possibility that systemic bone metabolism was within the normal range.

3- Although as claimed by the authors, the injection of anti-RANKL antibody may not protect against joint inflammation and cartilage destruction, the arthritis scoring (Figure 3) suggests that it would rather increase and may be initiate the disease. Indeed, in the presence of the antibody, the arthritis score seemed to worsened and even some sign of arthritis may be seen in the RA-/AB+ group, could the author comment on this? Can they provide an explanation? Did they performed long term experiment to see whether the scoring will be significantly raising?

Response:

We reanalyzed our RA score data carefully and found that we found errors in counting the score in RA-/Ab+ mice. There was no increase in the RA score in that group. Hence we revised Figure 3 correctly. We are very sorry for presenting the incorrect data in the old Figure 3. In addition, we added Supplementary Figure 1 that shows the results of time course study on paw thickness in 4 limbs of the individual mouse. There was no appreciable change in paw thickness in RA- mice irrespective of whether they were given OYC1 or not. Therefore, it seems unlikely that OYC1 anti-RANKL antibody has any activity to initiate or worsen arthritis.

4- I am not convinced by the histological pictures where it is very difficult to evaluate the presence of inflammation, for instance, the arrow on figure 4 O does not appear to point to inflamed area but rather on the meniscus. Could
quantitative date be provided for the inflammation and the cartilage destruction?

Response:

The arrowhead in Figure 4O might be somewhat difficult to point to the area of inflammation, whereas it does not point to the meniscus. We revised Figures 4O and 4P by moving arrowheads to point to the area of inflammation.

Response to Reviewer 2:

Thank you very much for reviewing our manuscript. Your comments and our responses to them are as follows.

1. Apparently, the authors have conducted the CAIA experiment only once with 5 to 6 mice per group. This number is too low to draw any conclusions (even for a negative result), especially with the extremely high standard variations of the RA+/Ab- group in Figure 3.

Response:

It is true that the standard variations especially in the RA+/Ab- group were high. However, not only statistical analysis of the effect of anti-RANKL antibody on the RA score, but the other data presented in Figures 4 and 5 as well as Supplementary Figure 1 indicate that anti-RANKL antibody did not have positive effect on inflammation and cartilage degeneration in arthritis.

2. In table 1 the authors describe that none of the RA- animals had swollen limbs. Yet in Figure 3, the RA-/Ab+ group develops an arthritis score from day 11, which is contradicting Table 1.

Response:

We reanalyzed our RA score data carefully and found that we had made mistakes in counting the score in RA-/Ab+ mice. We are very sorry for presenting incorrect data. Actually there was no increase in the RA score in that group. Hence, we have revised Figure 3 correctly. In addition, we added Supplementary Figure 1 that shows the results of time course study on paw thickness in 4 limbs of the individual mouse, which may assist understanding of the results shown in Table 1. We did not observe any appreciable swelling in the paws of RA- mice irrespective of whether they were given anti-RANKL antibody or not.

3. The scoring system of Figure 3 is not entirely clear. Did the authors use a cumulative score or did they calculate the mean of all paws?

Response:
They are calculated based on the criteria shown in the Materials and Method section. The values shown in Figure 3 are the mean of the scores from all mice in each experimental group. Data on swelling of each paw of individual mice are now presented in Supplementary Figure 1.

4. In Figure 2 the authors nicely demonstrate that the antibody against RANKL worked in the RA- mice by investigating the increase of the bone density in the tibia. However, this does not prove for 100% that the antibody also works in a more inflammatory environment. Would it be possible to provide these data also for the RA+ mouse groups?

Response:

Thank you for your suggestion. We revised Figure 2 by incorporating the results obtained in RA+ mice. Anti-RANKL antibody caused increase in bone volume (BV/TV) in RA+ mice.

5. In Figure 4 and 5 it would be nice to have a quantitative analysis of the amount of inflammation and cartilage destruction (for example a semiquantitative scoring system). In addition, HE staining is not the best staining to investigate cartilage destruction. A toluidine blue or safranin o staining would be much more appropriate.

Response:

According to your suggestion, we performed Safranin O staining of the articular cartilage. Yes, it was more appropriate to evaluate the cartilage degeneration (revised Figure 5). Unfortunately, quantitative analysis of inflammation was difficult because of the difficulty to get tissue slices of the same position in complicated structures of the joints from all the mice.

6. In Figure 5E the serum COMP of the RA- mouse groups should be included to show that the CAIA had an effect on the COMP levels.

Response:

According to your suggestion, we analyzed COMP in the sera from all the mice, and we found that the COMP level was not elevated significantly by injection of the cocktail of anti-type II collagen antibodies and LPS. We considered that COMP was not appropriate to evaluate degeneration of the articular cartilage in our model. Hence we have deleted the data on COMP in the revised manuscript.

Minor Essential Revisions:
1. In Figure 4, the labeling on the left side was not readable.

Response:
We have revised Figure 4 according to your suggestion. We hope the labels are readable now.