Author's response to reviews

Title: (5R)-5-hydroxytriptolide Inhibits Osteoclastogenesis through RANKL/RANK/OPG Signaling Pathway

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Author's response to reviews: see over
Dear Editor,
Thank you for giving us an opportunity to revise our manuscript. The point-to-point reply to the reviewer’s comments is as listed below. If any more question, please contact me again.

Sincerely yours,

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Reviewer: Kyung-Hyun Park-Min

Reviewer’s report:
This study examines the role of LDDT-8 on RANKL and OPG expression in CD3+ T cells isolated from PBMCs and SFMCs of rheumatoid arthritis patients. Authors also test the effect of LDDT-8 on RANKL-induced osteoclastogenesis of RAW cells. LDDT-8 is a compound isolated from the extracts of traditional Chinese medicine and has shown efficacy on various diseases. Author shows that LDDT-8 treatment on patients’ PBMC and SFMCs increases OPG expression and OPG/RANKL ratio in CD3+ T cells. LDDT-8 treatment also regulates cytokine profiles in patients’ PBMC and SFMCs and suppresses RANKL-induced osteoclastogenesis of RAW cells by inhibiting NF-KB activation. Data shown in the proposed manuscript are interesting but three parts of the data are not well linked in the manuscript.

- Major compulsory Revisions
1. Fig 1 and 2: The data in figure 1 and 2 are important points of the manuscript. The author should show the representative graph (either density plot or contour diagram) and mark the gate settings.

Reply: Thanks for the comments. The suggestion was useful. We added the pseudocolor plots to make the illustration easier to understand.

2. Fig 3 and 4: It would be useful to know the baseline of each cytokine in unstimulated samples and author should include the results from unstimulated samples. In addition, baseline cytokine profiles would determine the contribution of activated T cells on cytokine production.

Reply: Thanks for the comments. We agree with the reviewer that the baseline of each cytokine in unstimulated samples will be helpful to determine the contribution of activated T cells on cytokine production. However, it is hard for us to get unstimulated samples from healthy people, especially synovial fluid. In addition, the key point of Fig 3 and 4 was to demonstrate the effect of LLLDT-8 on cytokine production. Thus, we did not add the baseline data in the figures.
3. Fig 5: Osteoclasts are defined as TRAP-positive multinuclear (more than three nuclei) cells. It is difficult to determine the effect of LDDT-8 on osteoclastogenesis by current images. Improved photographic images are required. Either 10X or 20X magnification is better to determine the effect of LDDT-8 on osteoclast differentiation.

Reply: Thanks for the comments. We have changed the Fig 5 with more clear images.

4. Fig 7: Data will be more convincing if the author shows the graph of densitometry of pIkB/GAPDH.

Reply: Thanks for the comments. We added the relative quantification of the graph as Figure 7B.

- Minor Essential Revision
1. Fig. 5: Labels are missing.

Reply: Thanks for the comments. We added the labels.

Reviewer: Toru Yago
Reviewer's report:
Comments to the author
The authors aimed to clarify the mechanism of to assess the effects of LLDT-8 on RANK/RANKL/OPG signaling pathway and osteoclastogenesis. In the current report, they demonstrated that LLDT-8 up-regulated OPG expression in CD3+ T leukomonocyte, and increased the ratio of OPG to RANKL in peripheral blood of RA patients. They also showed that LLDT-8 increased the ratio of OPG/RANKL in synovial fluid of RA patients. Next, they also showed that LLDT-8 inhibited secretion of osteoclastogenic cytokines including IL-1β, IL-6, IL-21 and that LLDT-8 promoted secretion of anti-osteoclastogenic cytokine, IL-10, in the supernatants of PBMCs from peripheral blood or SFMCs from synovial fluid with RA patients. Furthermore, they demonstrated that LLDT-8 may reduce osteoclastogenesis by inhibiting NF-κB signaling. Finally, they concluded that LLDT-8 could have a therapeutic potential for RA. The author's studies are interesting; however, I have major and minor concerns on their study.

Major concern:
1. They estimated osteoclasts only by TRAP staining. They described that “Osteoclast formation was determined to be TRAP-positive staining multinuclear cells using light microscopy.” in Methods; however, we could not detect multinuclear osteoclasts in Figure 5. I could not understand how they made the graph of Figure 6 from Figure 5. I recommend that they evaluate osteoclasts also by immunostaining with anti-vitronectin receptor antibody. I also recommend that they evaluate the ability of bone resorption when they determine osteoclasts.
Reply: Thanks for the comments. In order to make the osteoclasts more clear, we repeated the experiment and got some improved photographic images. In the new images we can find the representative multinuclear cells easily (as shown in the revised Figure 5). Immunostaining and bone resorption assay are useful to evaluate osteoclasts, and we will try to use them to detect the function of osteoclasts in the further study.

Minor concerns:
1. They used “osteoclasia” two times in the current paper. We do not use this word usually. It is better to change to another word.
   Reply: Thanks for the comments. We changed “osteoclasia” with osteoclastogenesis in the revised manuscript.

2. In Background, the first paragraph is too long.
   Reply: Thanks for the comments. We simplified the background as shown in the revised manuscript.

3. In page 7, they used “didn’t” two times. Furthermore, in line 194, they described “To examine the effect of LLDT-8 on the expression on CD3+ T leukomonocyte in synovial fluid, we isolated SFMCs from synovial fluid of RA patients..”.
   There were two periods.
   Reply: Thanks for the comments. We have revised the sentences.

4. In Results, they did not describe the explanation about Figure 5C. I think it is the most important point in the current paper.
   Reply: Thanks for the comments. We added the explanation about Figure 5C in the Results section.

5. Throughout the paper, English should be revised by native speaker.
   Reply: Thanks for the comments. We polished the English expression throughout the paper.