nature portfolio

Peer Review File

Secretory leucocyte protease inhibitor regulates bone metabolism and inflammation in experimental mouse periodontitis

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript presents the role of SLPI in protecting against inflammation in periodontitis. In addition to being downregulated during periodontitis, the administration of SLPI in mice shows a strong inhibitory effect on periodontitis in a model of ligature-induced periodontitis. The authors link this effect of SLPI to several actions. First, SLPI inhibits osteoclast formation and activity along the alveolar bone, possibly through the downregulation of NFATC1, RANK, Acp5, and CtsK mRNA expression in osteoclasts. Results obtained from gingival tissue from mice are reminiscent of results obtained in vitro from cultures of osteoclasts treated with SLPI. In addition to its effects on osteoclasts, SLPI appears to strongly modulate osteoblast differentiation in vitro. Overall, this study is well-conducted and contributes to understanding the role of this inhibitor in periodontitis."
Please, find below my concerns:
1- The authors assert an upregulation of SLPI expression in the periodontal ligament. However, the images presented in Figure 1A are not clear. The two panels display different regions of the mice periodontal tissue. While the upper panel shows a large portion of the connective tissue surrounding the alveolar bone, the lower panel presents the periodontal ligament. This discrepancy in the regions depicted introduces bias into the comparison between the two images. To confirm this assertion, a proper quantification of SLPI protein expression via immunostaining in the gingival tissue should be conducted within the same region
2- Cause of its prolin-rich domains and di-sulfide bond, SLPI is a very difficult protein to produce in vitro. When produced in bacteria, several isoforms are found. A quality control needs to be added, with at least a Coomassie blue staining.
3- Figure 3, while a downregulation in Rank, Acp5 or CtsK reflects the decrease of osteoclasts in the gingival tissue, the involvement of Nfatc1 expression downregulation is less clear, as Nfatc1 is not specific of osteoclasts, and is expected to be expressed by several other cells. More generally, the impact of SLPI on other cells (especially the neutrophils) in the tissue is not explored here and the general features of inflammation are not analyzed. The targeting of neutrophils, as well as monocytes to the gingival tissue needs to be quantified.
4- Authors observe an alveolar bone loss up to 1.5 mm. This does not correspond to the images. To what exactly refers this measure?
5- In figure 4, while TRAP activity seems to be downregulated in the presence of SLPI, the expression of Acp5 is not. This discrepancy needs to be addressed in the manuscript.
6- The figure legends, as well as the material and method section should be more detailed for improved readability and better reproducibility. For example, NE activity measurement protocol gives no detail and no concentration of the elastase substrate.
7- Did the authors verify the normal distribution of their samples before performing an ANOVA?

Reviewer #2

(Remarks to the Author)
In this report, Sasagawa and colleagues have investigated the role of Secretory leukocyte protease inhibitor (SLPI) to gingival inflammation and bone loss by employing a mouse model of periodontitis and by performing in vitro experiments in macrophages.

Based on previously reported data on SLPI obtained from human sample analysis, SLPI may be a critical determinant in maintaining the homeostatic balance between proteases and anti-protease factors in the context of periodontitis. Therefore, findings of this report using a well-established model of ligature-induced periodontitis are interesting and set foundations for more detailed future studies focusing on the development of SLPI-based therapeutic approaches. However, addressing the following points would be required to ensure specificity of the presented findings and ultimately increase the impact of the study.

1. Besides the treatment with PBS, the authors are encouraged to use more relevant control treatments in comparison to SLPI treatment. Testing of additional control(s) should be performed in key in vivo and in vitro experiments. This experiment will exclude the possibility of any off-target effects.
2. Is the in vivo treatment with SLPI (Figure 2) expected to affect SLPI expression levels in the treated gingival tissue?
3. Figure 4d, i: It would be useful to determine the expression levels of these targets also after treatment with 10ug/ml of SLPI. This would confirm a dose-dependent role of SLPI on the regulation of gene expression.
4. Figure 2e and figure 4: Besides the investigation of pro-inflammatory genes and osteoclast-related factors, it is important to assess the levels of representative molecules involved in the resolution of inflammation and also determine the levels of markers exerting anti-inflammatory properties.
5. Figure 2: The combined treatment with SLPI and NE inhibitor should be tested to address any additive or synergistic effect.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have fully responded to all my comments and recommendations.

Reviewer #2

(Remarks to the Author)
All comments from reviewers were adequately addressed.

Response to Reviewer 1 Comments

We are grateful to Reviewer 1 for the critical comments and suggestions, which have helped us improve our paper considerably. We have considered all of the comments and suggestions in the revised version of our paper.

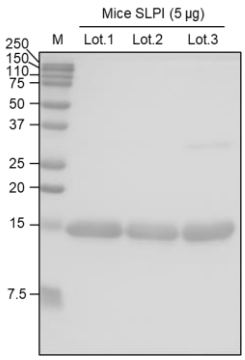
Point 1: The authors assert an upregulation of SLPI expression in the periodontal ligament. However, the images presented in Figure 1A are not clear. The two panels display different regions of the mice periodontal tissue. While the upper panel shows a large portion of the connective tissue surrounding the alveolar bone, the lower panel presents the periodontal ligament. This discrepancy in the regions depicted introduces bias into the comparison between the two images. To confirm this assertion, a proper quantification of SLPI protein expression via immunostaining in the gingival tissue should be conducted within the same region.

Response 1: Fig 1a and b (in the revised version)

Thank you for your comments. We have performed immunostaining for SLPI in the mouse periodontal tissue (Figure 1a) and calculated the fluorescence intensity (Figure 1b) in the same region.

Point 2: Cause of its prolin-rich domains and di-sulfide bond, SLPI is a very difficult protein to produce in vitro. When produced in bacteria, several isoforms are found. A quality control needs to be added, with at least a Coomassie blue staining.

Response 2: Thank you for your comment.



As you suggested, we have performed CBB staining using rSLPI from different lots and confirmed that the stained images were similar (left panel). In addition, because we used *Escherichia coli* Rosetta-gami B (DE3) strain, which allows for enhanced expression of eukaryotic proteins that contain codons rarely used in *E. coli*, to produce rSLPI, we obtained a full-length SLPI recombinant protein.

Point 3: Figure 3, while a downregulation in Rank, Acp5 or CtsK reflects the decrease of osteoclasts in the gingival tissue, the involvement of Nfatc1 expression downregulation is less clear, as Nfatc1 is not specific of osteoclasts, and is expected to be expressed by several other cells. More generally, the impact of SLPI on other cells (especially the neutrophils) in the tissue is not explored here and the general features of inflammation are not analyzed. The targeting of neutrophils, as well as monocytes to the gingival tissue

needs to be quantified.

Response 3: Fig. 3b (in the revised version) and lines 90-92.

Thank you for your comments. As you pointed out, *Nfatc1* is not a gene that is specifically expressed in osteoclasts. Therefore, we cannot conclude that the reason for the decrease in *Nfatct1* gene expression in mouse periodontal tissues, as shown in Figure 4, is that SLPI selectively inhibits osteoclast expression. However, *Nfatc1* is a master gene for osteoclasts, and we showed that *Nfactc1* gene expression was suppressed when SLPI was applied to mouse osteoclasts in Figure 5d. In addition, because it was difficult to isolate neutrophils and monocytes from mouse periodontal tissue, we demonstrated the anti-inflammatory effects of SLPI by analyzing the expression of *Il-6*, *Il-1b*, and *Il-10* in mouse periodontal tissue (Figure 3b). These findings suggest that intragingival administration of SLPI minimizes alveolar bone loss, downregulates the transcription of proinflammatory cytokine genes, and upregulates the anti-inflammatory cytokine gene IL-10. (Lines 90-92)

Point 4: Authors observe an alveolar bone loss up to 1.5 mm. This does not correspond to the images. To what exactly refers this measure?

Response 4: Thank you for the comment. The amount of bone resorption was measured using a dissecting

microscope and video image measurement system. According to a previously described method [42], the distance from the cement-enamel junction to the alveolar bone crest was measured at six predetermined sites on the ligated second molar and adjacent affected regions (left image). Bone changes were calculated by subtracting the sum of the cement-enamel junction and alveolar bone crest values in the ligated area from the six corresponding values in the unligated area. Negative values (mm) indicate bone loss relative to the baseline (unligated control). (Line 230-237)

Point 5: In figure 4, while TRAP activity seems to be downregulated in the presence of SLPI, the expression of Acp5 is not. This discrepancy needs to be addressed in the manuscript.

Response5: Lines 121-126 (in the revised version)

Thank you for your comment. Acp5 is a gene that is expressed during the middle to late stages of osteoclast differentiation. Since SLPI reduced the expression levels of the early transcription genes *Nfatc1* and *Rank*, it is possible that SLPI acts on osteoclasts in the early stages of differentiation, but not on osteoclasts that

have progressed to the middle to late stages of differentiation. In this regard, we have added the following sentences to lins 121-126 “Additionally, in the SLPI-treated group, the transcription of Nfatc1 and Rank was significantly downregulated when compared to that in the medium only group, whereas no significant differences were observed for Acp5 and Ctsk (Figure 5d). It has been reported that Acp5 and Ctsk are expressed in the terminal differentiation of osteoclasts and affect their bone resorption ability 25. These data suggest that SLPI inhibits the initial formation of osteoclasts.”

Point 6: The figure legends, as well as the material and method section should be more detailed for improved readability and better reproducibility. For example, NE activity measurement protocol gives no detail and no concentration of the elastase substrate.

Response 6: Lines 193-200, 215-222, and 226-229.
Thank you for your comments. As you suggested, we have added more details to the Materials and Methods section. The samples were centrifuged at 300×*g* for 3 min and NE activity in the supernatant was determined using *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide (Merck Millipore, Billerica, MA, USA). Samples were incubated in 0.1 M Tris-HCl buffer containing 0.5 M NaCl and 1 mM substrate at 37 °C for 24 h, and then absorbance at 405 nm was measured.” (Line226-229)

Point 7: Did the authors verify the normal distribution of their samples before performing an ANOVA?

Response 7: Thank you for the comment. In this study, we confirmed that the sample data generally showed a normal distribution by performing Shapiro-Wilk test.

Response to Reviewer 2 Comments

We are grateful to Reviewer 2 for the critical comments and suggestions, which have helped us improve our paper considerably. We have considered all of the comments and suggestions in the revised version of our paper.
Point 1: Besides the treatment with PBS, the authors are encouraged to use more relevant control treatments in comparison to SLPI treatment. Testing of additional control(s) should be performed in key in vivo and in vitro experiments. This experiment will exclude the possibility of any off-target effects.

Response 1: Fig. 2 and lines 156-159 (in the revised version).
Thank you for your comment. As an additional control, we have conducted additional experiments using BMP-2, which has been shown to promote bone formation in previous studies, and MR16-1, a selective inhibitor of the inflammatory cytokine IL6 (Figure 2). We also added the following sentences “In the present

study, we have investigated the effects of the administration of an anti-mouse IL-6 receptor antibody (MR16-1) 33 and a bone morphogenetic protein, BMP-2 25, on inflammation-induced alveolar bone resorption in a mouse periodontitis model. The administration of MR16-1 or BMP-2 and NE inhibitors reduced alveolar bone loss."(Lines 156-159)

Point 2: Is the in vivo treatment with SLPI (Figure 2) expected to affect SLPI expression levels in the treated gingival tissue?

Response 2: Fig. 3b and lines 92-95 (in the revised version).
Thank you for your comments. Figure 3b shows that SLPI administration increased *slpi* gene expression in mouse gingiva. We have also added the following sentences "Notably, the transcription of slpi also increased in mouse gingival tissues compared to that in the ligated + PBS group. These findings suggest that the intragingival administration of SLPI minimizes alveolar bone loss, downregulates the transcription of proinflammatory cytokine genes, and upregulates anti-inflammatory cytokine genes. (Line 92-95)"

Point 3: Figure 4d, i: It would be useful to determine the expression levels of these targets also after treatment with 10ug/ml of SLPI. This would confirm a dose-dependent role of SLPI on the regulation of gene expression.

Response 3: Fig. 5d (in the revised version)
Thank you for your comment. In Figure 5d, we also conducted additional experiments with the treatment of 10 μg/mL of SLPI. As a result, it was shown that the gene expression of *Nfactc1*, a master gene for osteoclast differentiation, was suppressed in a concentration-dependent manner by SLPI.

Point 4: Figure 2e and figure 4: Besides the investigation of pro-inflammatory genes and osteoclast-related factors, it is important to assess the levels of representative molecules involved in the resolution of inflammation and also determine the levels of markers exerting anti-inflammatory properties.

Response 4: Fig. 3b and lines 93-95 (in the revised version).
Thank you for your comments. We have added the results of the analysis of *IL-10* expression in Figure 3b). SLPI administration increased the gene expression of *IL-10,* an anti-inflammatory cytokine, *in vivo*. We also added the following sentence "These findings suggest that the intragingival administration of SLPI minimizes alveolar bone loss, downregulates the transcription of proinflammatory cytokine genes, and upregulates anti-inflammatory cytokine genes." (Line 93-95)

Point 5: Figure 2: The combined treatment with SLPI and NE inhibitor should be tested to address any additive or synergistic effect.

Response 5: Thank you for the comment. To date, the therapeutic effects of SLPI alone on periodontitis have not been analyzed. This is the first study to clarify the anti-inflammatory effects of SLPI on periodontal tissues and bone metabolism. Therefore, combined treatment with SLPI and NE inhibitors could be a topic for future studies