Pam2CSK4 (TLR2 agonist) induces periodontal destruction in mice

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Abstract: Lipoproteins are important bacterial immunostimulating molecules capable of inducing receptor activator of nuclear factor-κB (RANKL) and osteoclast formation *in vitro* and *in vivo*. Although these molecules are present in periodontopathogenic bacteria, their role in periodontitis is not known. In this study, we used Pam_2CSK_4 (PAM2), a synthetic molecule that mimics bacterial lipoprotein, to investigate the effects of lipoproteins on periodontitis in mice. C57BL/6 male mice were randomly divided into three experimental groups: 1) Negative control group: animals received vehicle injection; 2) Positive control group: animals received injection of *Escherichia coli* lipopolysaccharide (LPS); 3) PAM2 group: animals received PAM2 injection. All the injections were performed bilaterally every other day into the palatal mucosa between first and second molars. After twenty-four days, the animals were euthanized to assess alveolar bone volume (micro-CT), cellular and extracellular composition in the gingiva (stereometric analysis), and osteoclast numbers (TRAP staining). Treatment with either PAM2 or LPS induced gingival inflammation, as demonstrated by increased infiltration of inflammatory cells and enhanced angiogenesis, associated with a smaller number of fibroblasts and decreased extracellular matrix. Importantly, treatment not only with LPS but also with PAM2 resulted in a larger number of TRAP+ multinucleated osteoclasts and significant loss of alveolar bone. Collectively, our data demonstrate that PAM2 can induce gingival inflammation and bone loss in mice, broadening the avenues of investigation into the role of lipoproteins in the pathogenesis of periodontal disease.

Keywords: Bone and Bones; Inflammation; Lipoproteins; Toll-Like Receptors; Mice; Periodontal Diseases; Osteogenesis.

Introduction

Periodontitis is a polymicrobial disease characterized by the host inflammation-dependent destruction of periodontal structures, leading to long-term alveolar bone loss and loosening of teeth.[1] The host response is initiated by the recognition of pathogen-associated molecular patterns (PAMPs), such as the lipoproteins and lipopolysaccharides (LPS) present in Gram-negative bacteria.[2,3] The immune system recognizes and responds to the PAMPs through different pathogen recognition receptors (PRRs) such as Toll-like receptors (TLRs), leading to up-regulation of a wide variety

of inflammatory mediators, and to a consequential antimicrobial response.[4,5,6,7] Recent findings in mouse models point out the possible transfer of a dysbiotic oral microbiome across individuals and generations, thereby highlighting the importance of identifying bacterial signatures and host receptors that could contribute to the pathogenesis of periodontitis.[8]

Animal models have been used to help understand the mechanisms involved in the pathogenesis of periodontitis, and provide important information on the inflammatory response associated with host-microbial interactions.[9,10,11,12,13,14,15,16,17] The main advantage of experimental models is that they allow the specific mechanisms involved in the pathogenesis of periodontitis to be studied in a more targeted and controlled manner.[10,11] The possibility of genetic modifications makes mice an excellent model to study the host response in periodontitis, and enables a better understanding of the role of different proteins and receptors in the recognition of microbes.[9] On the other hand, the use of genetically modified bacteria and purified bacterial molecules helps us understand the role of specific PAMPs in the initiation of an immune response.[9,18,19]

Its ubiquitous presence in Gram-negative bacteria makes LPS the most widely studied PAMP, and several studies show the importance of LPS-induced activation of TLR4 for periodontal breakdown.[9,12,20] Many bacteria also contain lipoproteins as important virulence factors, caused by their strong immunostimulatory activity.[21] *Porphyromonas gingivalis* (*P. gingivalis*) is recognized as an important pathogenic bacterium in human periodontitis. These bacteria induce gingival inflammation and periodontal breakdown by activation of TLR2 in experimental models of periodontitis in mice.[22] However, the bacterial components isolated from *P. gingivalis* are not the ideal tools to study TLR2-dependent periodontitis, since *P. gingivalis* LPS may present large variations among companies and batches, because this bacteria is able to modify its PAMPs in order to escape the immune system.[1,23,24]

The synthetic lipopeptide Pam_2CSK_4 (PAM2) mimics bacterial lipoprotein and binds specifically to TLR2, preferentially to the TLR2/6 heterodimer.[25] Previous reports demonstrated that PAM2 is capable

of inducing bone resorption through osteoclast activation. The mechanisms by which PAM2 induces osteoclastogenesis involve both direct stimulation of osteoclast precursors and product[22,26] In an effort to understand how TLR2 induces periodontitis, the authors compared the effect of PAM2 with that of a well-characterized *Escherichia coli* LPS.[9,10,12]

Methodology

Animal care

Mice and surgical procedures were handled according to the guidelines of the local Ethical Committee for Animal Care and Use (CEUA #11/2015). The animals were kept on the animal facilities of the São Paulo State University (UNESP), School of Dentistry, Araraquara, (Araraquara, Sao Paulo, Brazil), at a controlled temperature ($23^{\circ}C \pm 2^{\circ}C$) and a 12-hour light/dark cycle. Mice were housed in plastic cages, fed a standard laboratory diet, and given water *ad libitum*.

LPS and PAM2 Injection Model

A total of twenty-one 10-week-old C57BL/6 male mice with an average weight of 25 g were used in this study. The animals were sedated with isoflurane (Cristália, Itapira, Brazil), and received 3-µL local injections using custom-designed 0.375-inch x 12-gauge needles attached to a 10-µL syringe (Hamilton Company, Reno, USA). The injections were administered into the palatal mucosa between the first and second maxillary molars, and the animals were randomly divided to receive the following substances: a) Negative control group (CTR): phosphate-buffered saline (PBS); b) Positive control group (LPS): 5 µg of *E. coli* LPS (Lipopolysaccharide from *Escherichia coli* 0127:B8; Sigma Aldrich, St. Louis, USA); c) Experimental PAM2 group (PAM2): 5 µg of Pam_2CSK_4 (InvivoGen, San Diego, USA; synthetic diacylated lipoprotein – TLR2/TLR6 ligand). All the injections were given three times a week for 24 days.

At the end of the experimental period, the animals were euthanized, and their maxillae were fixed in 4% paraformaldehyde for 24 hours, and stored in 70% ethanol to perform the analyses described below.

Micro-computed tomography scanning (μCT scanning)

Bone samples were carefully harvested to evaluate bone destruction after periodontal disease induction. Previously, the maxillae were washed in distilled water, and wrapped in moist paper to prevent artefacts caused by dehydration during the scanning process. A high-resolution micro-CT imaging system (μCT Skyscan 1176; Skyscan, Kontich, Belgium) was used with the following parameters: 18 μm isotropic space resolution, X-ray generator at 57kVp, beam current at 184 μA, and 0.5 mm aluminum filter. The following parameters were applied to reconstruct the samples using an appropriate software program (NRecon, v.1.6.1.5, Skyscan, Kontich, Belgium): defect-pixel masking: 10%; beam hardening: 20%; smoothing: 1; and individually calculated misalignment compensation values. The images were imported into specific software (Data Viewer, v.1.4.3.1, Skyscan, Kontich, Belgium) to generate multiplanar reconstructed images, and coronal datasets were saved for further volumetric analysis. Bone volume fraction (BV/TV) analysis was assessed using appropriate software (CT-Analyser software v1.10.1.0; Skyscan, Kontich, Belgium), as previously described.[27,28] Briefly, a region of interested (ROI) with a cone frustum shape was manually delimitated, and 70 consecutive coronal slices were measured with a ROI extending from the root apices to the alveolar bone crest (ABC), and from the mesial root of the first molar to the distal root of the third molar, including the trabecular bone of the maxillae alveolar ridge, and excluding the periodontal ligament and teeth roots. Scans were de-identified and periodontal bone loss was measured by a blinded and experienced examiner (FACM).

Stereometric analysis

After scanning, the same samples were decalcified in ethylenediaminetetraacetic acid (EDTA) (0.5 M, pH 8.0) for 4 weeks at room temperature, and embedded in paraffin. Seven hemi-maxillae per group were used to evaluate morphologic changes. Serial parasagittal sections (5 μm) were obtained on the buccal-palatal plane (transversal), mounted on slides and stained with hematoxylin & eosin. Two sections of each tooth were selected for stereometric analyses. Images were captured using a digital camera (Leica DFC 300, Leica - Reichert Diastar Products & Jung, Wetzlar, Germany) coupled to an optical microscope (Leica DMLS, Leica - Reichert Diastar Products & Jung, Wetzlar, Germany) set at 200x magnification. A 17,765 μm² grid with 9 x 4 30-μm squares (in scale with the 40x magnification used in all the images) was constructed using image editing software (Adobe Photoshop CS6, Adobe System Incorporated, San Jose, USA) and overlaid on the digital images as described elsewhere.[29] The ROI was delimitated by the whole grid, which was positioned on the connective tissue, associated with the supra-crestal area at the palatal bone. A blinded and calibrated examiner (GJPLO) performed the stereometric analysis using a point-counting technique in a two-dimensional plane of the grid, to match the tissue components with the points of intersection. Cell type identification (inflammatory or fibroblast) was based on the morphological characteristics of the cell. Inflammatory cells usually have a rounded shape, whereas fibroblasts are elongated/fusiform. Although not all elongated cells are defined as fibroblasts, the authors of the present study called this type of cell as non-inflammatory. Blood vessels and other structures (called the extracellular matrix) were quantified, as described elsewhere.[29,30,31] The structures were evaluated using a light microscope at a higher magnification to ensure accurate recognition, and thus resolve any doubt by the investigator. If any doubt remained, the structure was recorded as 'extracellular matrix.' The presence of each structure was expressed as a percentage of the total points analyzed in the total area, as previously described.[29]

TRAP staining

Sections were stained using a TRAP staining kit (Leukocyte Acid Phosphatase 387-A, Sigma-Aldrich, St. Louis, USA) to identify the presence of osteoclast. Sections were deparaffinized and rehydrated, fixed in a fixative solution for 30 seconds, and then incubated at 37°C in TRAP staining solution (containing diazotized fast garnet, naphthol AS-BI phosphate, acetate, and tartrate solution) for 60 minutes, as described elsewhere.[32,33] After counterstaining with Harris hematoxylin, sections were dehydrated

and cover-slipped. The total number of TRAP+ multinucleated cells containing 2 or more nuclei was counted in the palatal and buccal side of the maxilla, in a total area of 245,877 μm^2. Analysis was performed on a total of 7 mice per group, by a blinded and experienced examiner (R.S.M.).

Statistics

Analyses were performed using GraphPad Prism Software (Graph-Pad Prism Software, La Jolla, USA). Group measurements were expressed as mean ± the standard error of the mean (SEM). The Shapiro-Wilk test confirmed the normal distribution of all data (p > 0.05). Statistical significance was assessed using one-way analysis of variance (ANOVA) followed by post hoc Tukey's test for multiple comparisons among groups. Differences were considered significant at p < 0.05.

Results

PAM2-induced gingival inflammation decreases alveolar bone mass

The general effects of the LPS and the PAM2 injections in the palatal aspect of the maxilla were evaluated by measuring the BV/TV of the alveolar ridge. The micro-CT analysis revealed that the alveolar bone architecture in the CTR group was maintained (Figures 1A and 1D). Alveolar bone resorption was seen in the LPS and PAM2 injection groups, and was statistically higher compared to that of the CTR group (p < 0.0001) (Figures 1B, 1C and 1D). The LPS group was characterized by more severe alveolar bone resorption than the PAM2 group (p < 0.05) (Figure 1D).

PAM2 induces gingival inflammation and breakdown of the extracellular matrix

Histologic stereometric analysis of the maxillae was performed to assess the effects on the cellular and extracellular matrix composition (Figures 2A-C1 and Figures 2D-G). PAM2 treatment resulted in a greater proportion of inflammatory cells in PAM2 (39.62 ± 2.45%), compared to the vehicle-treated controls (p < 0.0001) (11.14 ± 8.98%). The treatment also enhanced the proportion of vascular structures (p < 0.05) (14.47 ± 5.46%), compared with the vehicle-treated controls (10.66 ± 2.93%). The inflammatory response was associated with a smaller number of gingival non-inflammatory cells (p < 0.0001) (5.71 ± 3.26%) (Figures 2A-C1 and Figures 2D-E), compared with the controls (24.38 ± 4.30%), and decreased extracellular
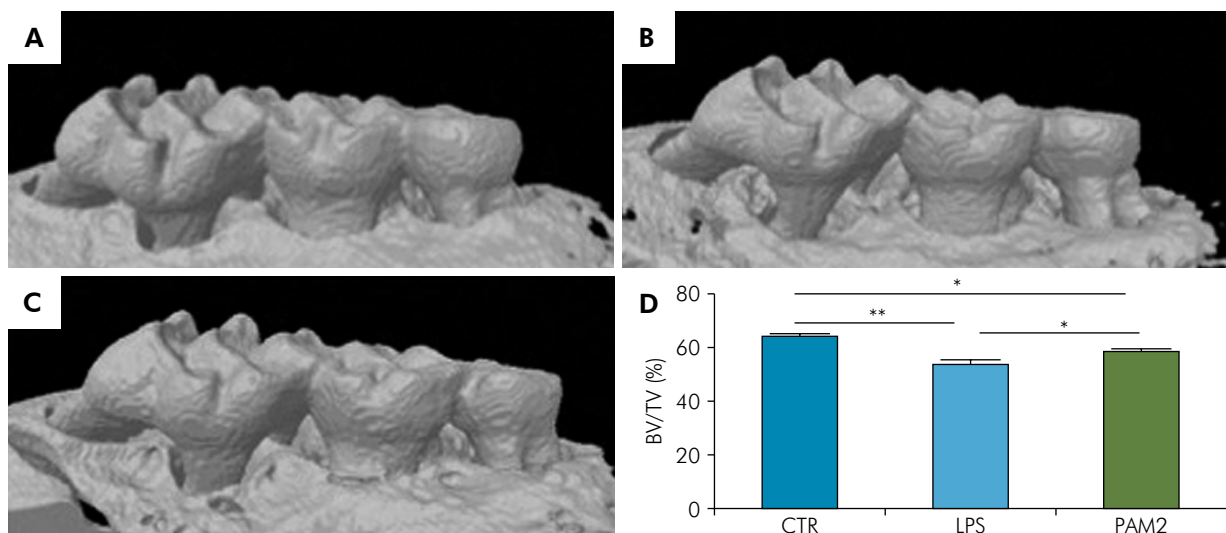
*Statistically significant difference from the indicated group p < 0.05; **Statistically significant difference from the indicated group p < 0.0001. Differences among groups were calculated by one-way ANOVA followed by Tukey's test. Data represent the mean ± SEM.

Figure 1. Microtomographic analysis of alveolar bone. (A-C): CTR group injected with saline solution (n = 7); LPS group injected with 5 μg LPS (n = 7); and PAM2 group injected with 5 μg PAM2 (n = 7) three times a week, for 24 days, in the gingival papilla in the palatal aspect, between the first and second molars, respectively; (D): Quantification of BV/TV in the maxilla of all mice.

matrix (p < 0.0001) (41.52 ± 5.37%), compared with the controls (56.24 ± 4.66%). Similar observations were made in mice treated with LPS.

PAM2 stimulates osteoclast formation

The effect of PAM2 on osteoclast numbers was determined using TRAP staining of histological sections to investigate if the decrease in bone mass induced by PAM2 was due to increased bone resorption (Figures 3A1-C3 and 3D). PAM2 treatment resulted in a significant increase in the number of TRAP+ multinucleated cells attached to the bone surface, with extended bone contact surface, compared with the vehicle-treated control group (p < 0.001) (Figure 3D). As expected, LPS treatment was also associated with a larger number of TRAP+ multinucleated osteoclasts (p < 0.0001).

Discussion

Several bacterial components and cellular pathways are involved in the pathogenesis of periodontitis. For this reason, there is no complete animal model that embraces all aspects of the disease in humans. Different
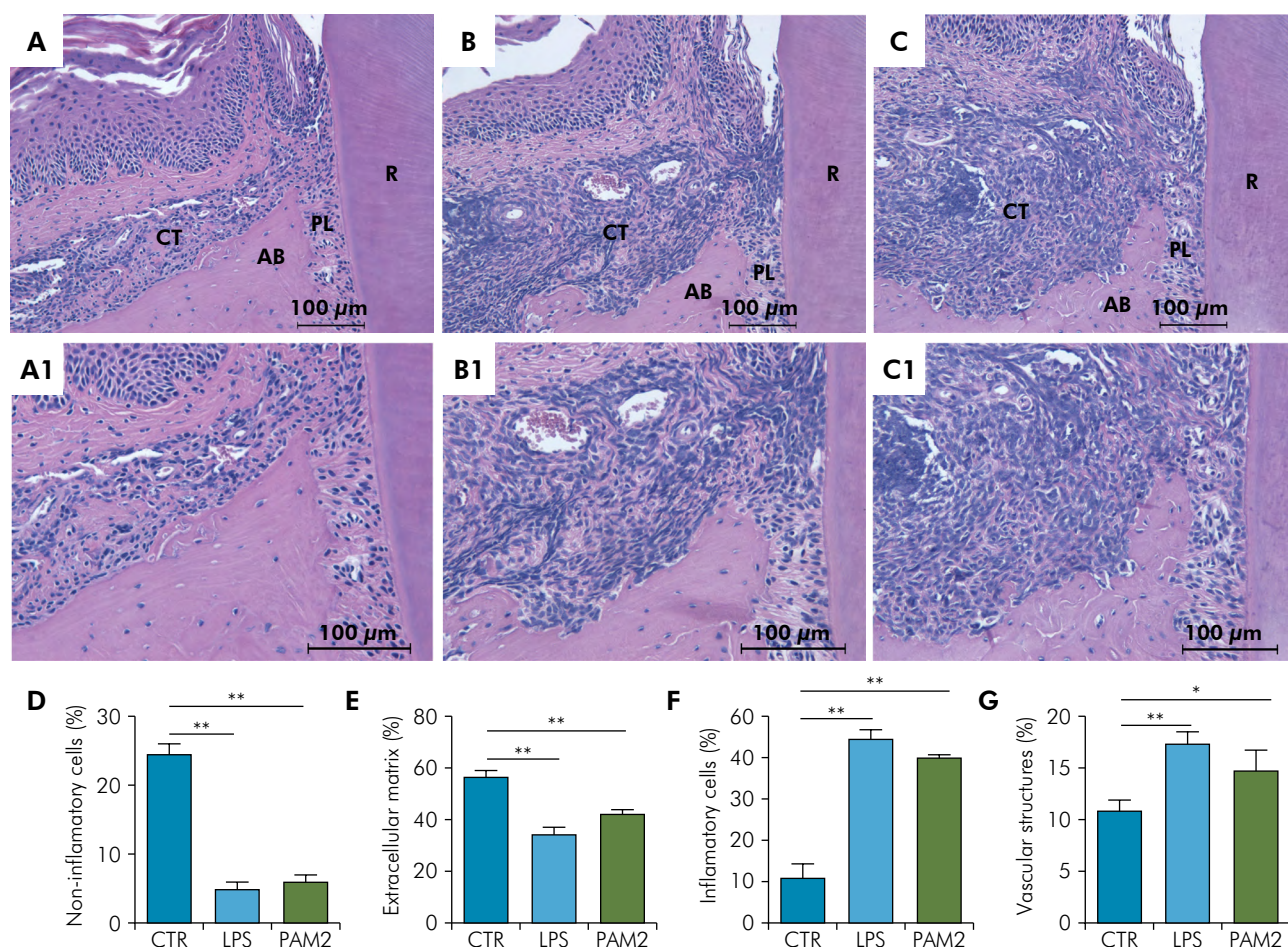
AB: alveolar bone; CT: connective tissue; PL: periodontal ligament; R: root. *Statistically significant different from the indicated group p < 0.05; **Statistically significant difference from the indicated group p < 0.0001. Differences among the groups were calculated by one-way ANOVA followed by Tukey's test. Data represent the mean ± SEM.

Figure 2. Representative H&E-stained images from the maxilla, and quantification of stereometric findings: (A-C): CTR (n = 7), LPS (n = 7) and PAM2 (n = 7) groups, respectively. Original magnification, 200x. (A1-C1) CTR, LPS and PAM2 groups, respectively. Original magnification, 400x. (D-G): Quantification of relative presence of fibroblasts, extracellular matrix, inflammatory cells, and vascular structures.
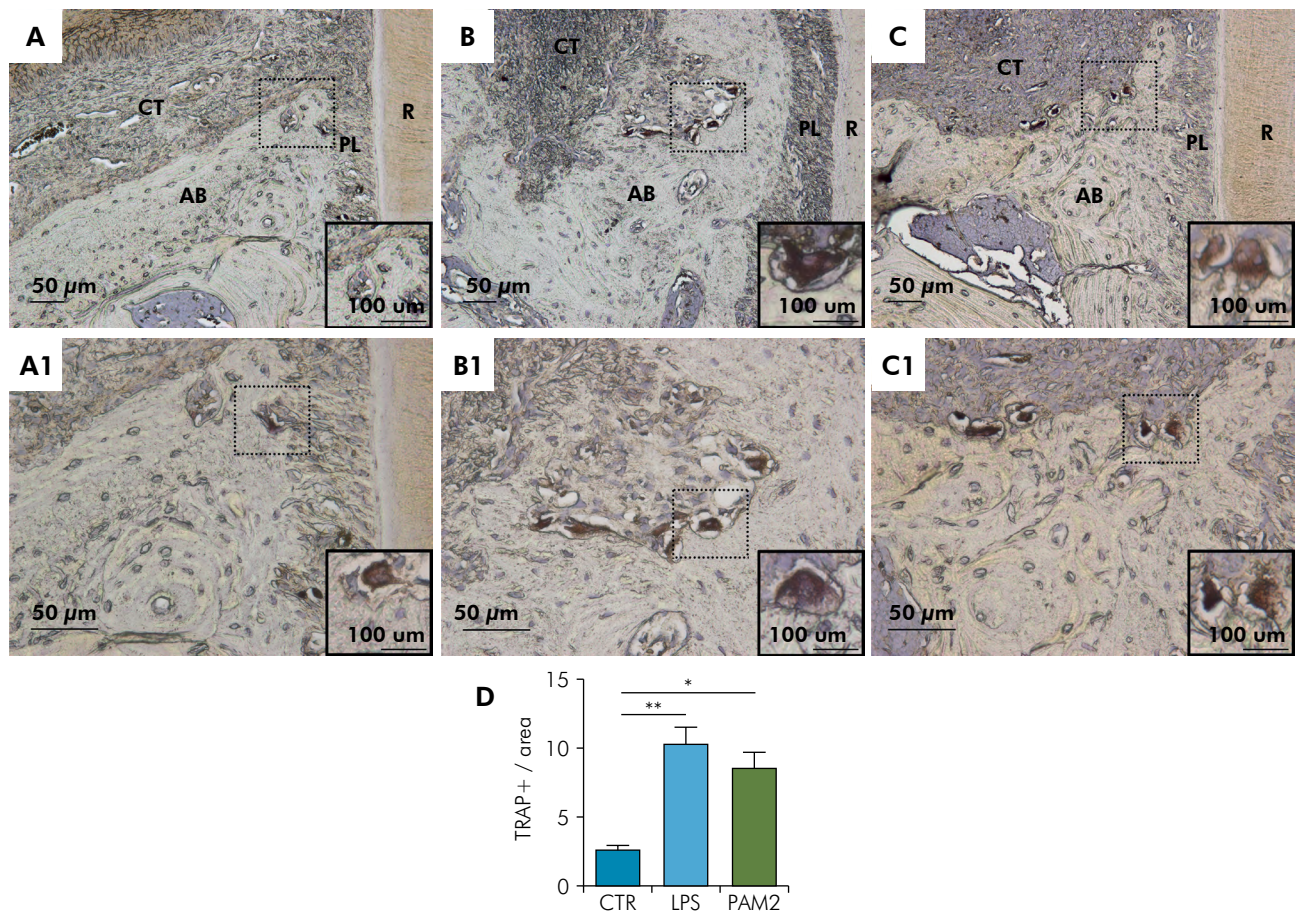
AB: alveolar bone; CT: connective tissue; PL: periodontal ligament; R: root. *Statistically significant difference from indicated groups, p < 0.001. **Statistically significant difference from indicated groups, p < 0.0001. Differences among groups were calculated by one-way ANOVA followed by Tukey's test. Data represent the mean ± SEM.

Figure 3. Counting of TRAP+ multinucleated cells. (A-C): Control (n = 7), LPS (n = 7) and PAM (n = 7) groups respectively. Original magnification, 20x. (A1-C1): Magnified views of the control, LPS and PAM groups. Original magnification, 40x. Insets are magnified views of individual TRAP+ cells in all groups; (D): Quantification of TRAP+ cell number in all mice and groups (n = 7 mice per group).

methods have been used to induce periodontitis in rodents, including gavage, bacterial infection, ligature and LPS injections.[10,13,14,17,29,34,35] The selection of a specific model depends on the aspects of the disease that will be focused on.[9] Each model can mimic some components of the pathological process during the course of periodontal disease. By selecting the appropriate model to test a specific hypothesis, each component can be investigated individually.[9] In studying the complex aspects involved in the pathogenesis of periodontitis, the animal models offer a vast field for exploration, by promoting a better understanding of the interaction between different bacterial components and the periodontal tissues of the host.[10,11,36]

A localized injection of bacteria or pathogenic component directly into the palatal gingival tissue is a widely used model to study periodontal disease.[37,38] This method causes significant periodontal inflammation, leading to the expression of a large number of inflammatory mediators that lead to bone loss near the area of the injection.[39,40] Furthermore, the injection of synthetic components allows control of the stimulus intensity, the vehicle selection and the substance volume, independent of variables such as colonization and survival of exogenous bacteria. The origin of the bacterial component used is the main variable to be considered, since different PAMPs activate different TLRs. Among these

receptors, TLR4 recognizes lipid A in LPS, whereas TLR2 recognizes several microbial components, including lipoteichoic acid, peptidoglycans, and lipoproteins.[4] TLR activation triggers the production of inflammatory cytokines by activating several signaling pathways, including MAPK and NF-κB, leading to inflammatory response and bone resorption.[27,41,42,43]

The characterization of lipoproteins as potent inducers of bone resorption[25] and the identification of these molecules as constituents of periodontopathogenic bacteria[19,22,26] led us to investigate the ability of PAM2 to induce periodontal disease. In the present study, the authors demonstrated that PAM2 is as potent as LPS in inducing inflammation and periodontal bone loss in mice.

Our data corroborate those of previous studies reporting the ability of periodontopathogenic bacteria to induce alveolar bone loss by activating TLR2[18,44] Interestingly, TLR2^{-/-} are more effective in bacterial clearance, and are protected from periodontal bone loss induced by *P. gingivalis.*[44] Further evidencing the importance of TLR2 for periodontal disease, TLR2 deficient mice are protected from alveolar bone destruction caused by *Tannerella forsythia.*[45] Although the previous data clearly demonstrate the importance of TLR2 in the pathogenesis of periodontal bone loss, the direct stimulation of periodontal disease using lipoprotein, to the best of our knowledge, has not yet been previously reported.

In the present study we choose to use a TLR4 agonist (*Escherichia coli* LPS) as a positive control instead of using a periodontopathogenic bacteria such as *P. gingivalis, Tannerella forsythia* or *Treponema denticola*. These common periodontopathogenic bacteria species, referred to as the "red complex," and closely related to periodontal disease, are known to induce bone resorption by activating the TLR2. This was the reason why LPS derived from *E. coli* (a non-periodontopathogenic species) was used as a positive control, namely, because it stimulates TLR4. Furthermore, LPS from *Escherichia coli* usually leads to an intense inflammatory process in the connective tissue and to bone resorption, each of which plays an important role as a positive control. The literature reports that both TLR2 and TLR4 are expressed by bone cells, and once these receptors are activated by their ligands, namely lipoproteins and LPS, they can stimulate bone loss, during the course of inflammatory processes.[3,22,42,43] We found that both molecules could promote well-located inflammatory bone loss by micro-CT and histological analyses. Compared with the negative control group, both LPS and PAM2 groups demonstrated a similar potential for periodontal disease induction. These findings corroborate previous findings showing osteoclast differentiation leading to severe bone loss *in vivo* when PAM2 was injected in mice.[22,26]

In our study, intense inflammation and alveolar bone loss in the periodontal tissue of animals stimulated with *Escherichia coli* LPS and PAM2 showed the potential involvement of both TLR2 and TLR4 in both the inflammation induction and the progression of periodontitis. As expected, these features were not evidenced in the CTR group, which was characterized by absence of tissue inflammation and bone resorption. Furthermore, the number of TRAP+ cells observed in the PAM2 group confirms the participation of TLR2 in *in vivo* osteoclastogenesis, which might explain the greater bone resorption in the animals receiving PAM2 injections. The similarity in the radiographic and histological characteristics of LPS and PAM2 closely resembles the features of human periodontitis, validating this approach as one that captures the essential [46,47]

Conclusion

Our findings indicate that continued injections of PAM2 into the palatal mucosa were an effective and reproducible model to induce gingival inflammation and alveolar bone loss in mice. This model can provide a new tool to investigate the specific role of TLR2 during the course of periodontal disease, considering that these mechanisms are not yet completely understood.

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