Induction of M2 Macrophages Prevents Bone Loss in Murine Periodontitis Models

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Appendix

Appendix 1

The aqueous solution was added into the oil phase, followed by 10 seconds of sonication at 25% amplitude, which is the first water-in-oil emulsion step. The second water-in-oil emulsion step was done by homogenizing (FSH125; Fisher Scientific) the sonicated solution in 60 mL of 2% (wt/vol) polyvinyl alcohol (P8136-250G; Sigma-Aldrich) for 60 seconds at 3,000 rpm. Subsequently, the solution was mixed with 80 mL of 1% polyvinyl alcohol, and stirred on an agitator for 3 hours at 600 rpm, allowing PVA to evaporate along with dichloromethane. The microparticles were then collected and washed four times with deionized water, and the final solution was lyophilized for 48hours in a LABCONCO FREEZONE 4.5 freeze dryer (Labconco).

Appendix 2

Pg was cultured under anaerobic conditions on Brucella blood agar supplemented with 5% blood, hemin and vitamin k1 (Remel^{TM}, Thermo Fisher Scientific). To inoculate bacteria in the mouse oral cavity, *Pg* was scraped from growth plates and resuspended at $1x10^{11}$ cfu in BBL brain heart infusion (BHI) agar (BD Biosciences) containing 2% carboxymethylcellulose (CMC). Before bacterial inoculation, the oral flora in mice was reduced by adding 15 mL/L of

Sulfatrim pediatric suspension (sulfamethoxazole and trimethoprim, 2 mg/mL wt/vol and 0.4 mg/mL wt/vol; Henry Schein) to their normal drinking water. After 7 days of antibiotic water, clean drinking water was switched back for 3 days, to reduce any potential antibiotic microbicidal effects on *Pg*. 50 µl of *Pg* BHI solution was given to each animal through oral gavage needle at a 48h interval for three times in the first week of the experiment to induce periodontitis.

Appendix 3

The standards for reorientation were that 1) horizontal plane came through the CEJ of first and second molars, and 2) sagittal plane came through the center of the mesial root canal of first molar and root canal of third molar. The distance between CEJ and ABC was measured by ImageJ on frontal sections after reorientation. Along mesial to distal direction on frontal sections, the CEJ-ABC distance of buccal and lingual side was measured at a 52.5 µm interval. In addition, the interdental CEJ-ABC distance was measured on sagittal sections at a 42 µm interval in bacterial model and 21 µm interval in ligature model. In the bacterial model, for both buccal and palatal side, 24 sites were measured for first molar (M1), 10 for second molar (M2) and 4 for third molar (M3). Twenty-eight interdental sites were measured in each sample as well. In the ligature model, the ROI of bone loss evaluation was the area surrounding second molar (M2). Twenty sites were measured for the buccal side of the second molar (M2), 20 for the palatal side, and 22 for interdental.

Appendix 4

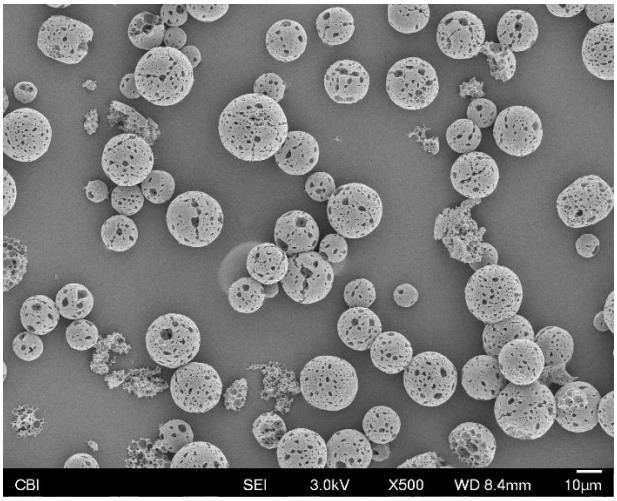
For each sample, near the buccal-lingual center, 3 sections with a distance ladder of 25-30 μm was stained to determine osteoclast numbers. TRAP-positive cells appeared as red cytoplasm and purple nuclei. In the *Pg*-induced periodontitis model, the area between alveolar bone crest to 0.24 mm beneath the root apex, 0.2 mm extension to the mesial end of M1 and distal end of M3 was considered region of interest (ROI). In the ligature model, ROI was defined as the upper 2/3 of the mesial and distal M2 alveolar crest, plus furcation area of M2. Within the ROI, all multinucleated TRAP-positive cells on alveolar bone surface or inside bone were counted under microscope. The surface area of alveolar bone within the ROI was measured with ImageJ.

Appendix 5

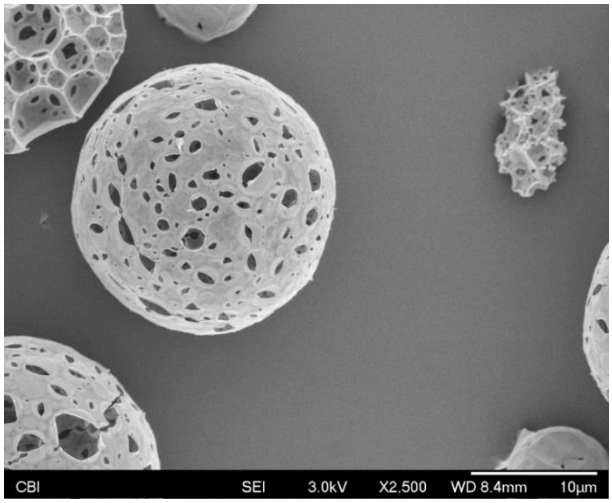
Sagittal section slides were deparaffinized and blocked by 2% donkey serum (Santa Cruz) for 1h at RT, then stained with goat-anti-mouse CD206 antibody (Cat: AF2535, R&D Systems) at 4 °C overnight, donkey-anti-goat IgG Alexa Fluor 594 secondary antibody (Invitrogen), and DAPI. The adjacent section underwent the very same procedure, without anti-CD206 antibody incubation, and served as negative control. For F4/80 antibody (Cat: MCA497R, AbD Serotec) staining, the slides underwent antigen retrieval in proteinase K buffer for 3 minutes at room temperature right before donkey serum blocking, and the rest of the staining procedure was the same as CD206 antibody staining. Cells in the interdental gingival papilla were counted with ImageJ, after pictures were taken with a fluorescent microscope (Eclipse TE2000-E, Nikon Instruments). Specifically, CD206 was used to determine the number of M2-phenotype macrophages, and F4/80 for pan-macrophages (M1 and M2). DAPI was used to locate gingival papilla. The percentage of M2 macrophages in interdental gingival papilla was determined by

the ratio of CD206+ cells to DAPI-stained cells in the area, and the percentage of total macrophages was the ratio of F4/80+ cells to DAPI-stained cells. Hence the difference between the M2 and pan-macrophage percentage provided us the M1-phenotype macrophage percentage in the papilla. We then calculated the M1/M2 ratio.

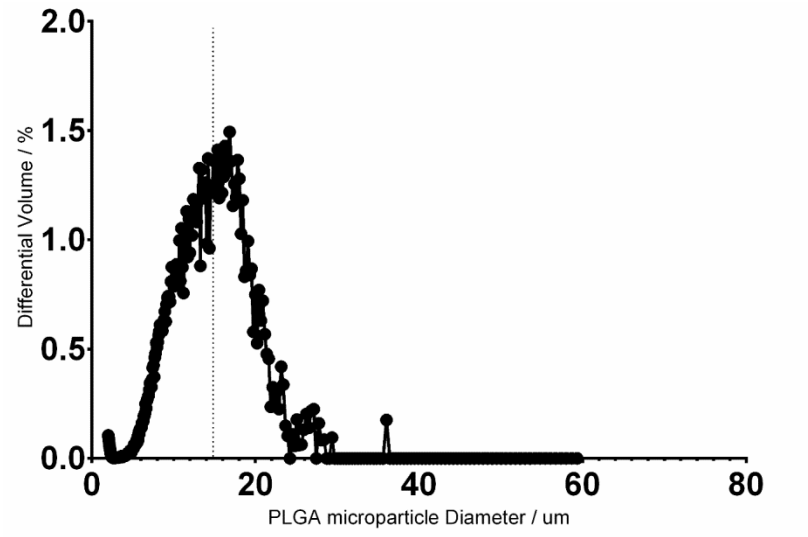
a



b



c



Appendix Figure. Characterization of CCL2-releasing PLGA microparticles.

Scanning electron microscope (SEM) images of CCL2 PLGA microparticles, a) 500x magnification and b) 2500x magnification respectively. CCL2 PLGA microparticles are spheres, with pores on the surface and inside. CCL2 microparticles size distribution. c) Volume impedance test by Beckman Coulter counter, the CCL2-releasing MPs have an average particle diameter of 14.44µm (dotted line) with ±5.22µm SD.