Supporting Information

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SI Materials and Methods

Fabrication, Characterization, and Administration of C-C Motif Chemokine Ligand 22 Microparticles. Poly(lactic-co-glycolic) acid (PLGA) microparticles containing recombinant mouse (for mouse investigations) or recombinant human (for canine investigations) C-C motif chemokine ligand 22 (CCL22; R&D Systems) were prepared by using a standard water-oil-water double emulsion procedure as described previously (1). Blank (unloaded) PLGA microparticle controls were fabricated in the same manner with the exception of CCL22 protein encapsulate. Briefly, the PLGA (RG502H; Boehringer Ingelheim) microparticles were prepared by mixing 200 μL of an aqueous solution containing 5 μg of recombinant mouse CCL22 [25 μg recombinant human CCL22 (rhCCL22) for canine investigations] and 2 mg of BSA and 15 mmol NaCl (to create pores, 5 mmol NaCl was used in rhCCL22 microparticle preparation) with 200 mg of polymer dissolved in 4 mL of dichloromethane. The first water-in-oil emulsion was prepared by sonicating this solution for 10 s. The second oil-in-water emulsion was prepared by homogenizing (L4RT-A; Silverson) this solution with 60 mL an aqueous solution of 2% (wt/vol) polyvinyl alcohol (molecular weight ∼25,000 g/mol, 98 mole % hydrolyzed; PolySciences) for 60 s at 3,000 rpm. This solution was then mixed with 1% polyvinyl alcohol and placed on a stir plate agitator for 3 h to allow the dichloromethane to evaporate. The microparticles were then collected and washed four times in deionized (DI) water to remove residual polyvinyl alcohol before being resuspended in 5 mL of DI water, frozen, and lyophilized for 72 h (VirTis BenchTop K freeze dryer; operating at 100 mTorr).

Surface characterization of microparticles was conducted by using scanning EM (JSM-6330F; JEOL), and microparticle size distribution was determined by volume impedance measurements on a Beckman Coulter counter (Multisizer-3; Beckman Coulter). CCL22 release from microparticles was determined by suspending 7 to 10 mg of microparticles in 1 mL of PBS solution placed on an end-to-end rotator at 37 °C. CCL22 release sampling was conducted at various time intervals by centrifuging microparticles and removing the supernatant for CCL22 quantification by using ELISA (R&D Systems). Microparticles were resuspended with 1 mL of fresh PBS solution and returned to the rotator at 37 °C.

For mouse investigations, microparticles were administered to four sites via 2% carboxymethylcellulose (CMC) in PBS suspension. Specifically, 2 to 5 μL of solution containing 25 mg/mL of particles were administered to the proximal side of the first molar, each interdental site, and distal to the third molar of the right maxilla of the mice. Microparticles were injected into maxillary gingiva of mice by using 27- to 28.5-gauge insulin syringes. For C57BL/6 mice inoculated with *Actinobacillus actinomycetemcomitans* (*Aa*), microparticles were injected on days −1, 10, and 20 relative to the first bacterial inoculation. For BALB/c mice inoculated with *Porphyromonas gingivalis* (*Pg*), microparticles were injected on days −1, 20, and 40 relative to the first bacterial inoculation. Microparticles were injected in mice at a depth of ∼100 to 300 μm within the maxillary gingival tissues. All microparticle injections in mice were preformed under a stereomicroscope. Small amounts of the microparticle solution were observed to overflow into the oral cavity of the mice during injections. Dogs received ∼2 to 4 mg of dry particles to the subgingival pockets (periodontal pocket) of the fourth premolars and carnassial (first molar) of both mandibles. Dry microparticles were deposited in the subgingival pockets by using empty, cleaned, and refilled Arestin injector tips. The dry microparticles hydrated (in gingival crevicular fluid of the dogs) and swelled immediately, retaining them within the periodontal pocket of dogs, and we observed no microparticle leakage from the pockets.

Periodontal Disease Induction. ***Mouse.*** The mouse model for periodontitis was conducted as described previously (2, 3). Briefly, WT male C57BL/6 mice aged 8 wk were purchased from Charles River Laboratories International or bred and maintained in the animal facilities of the Department of Biochemistry and Immunology at Faculdade de Medicina de Ribeirão Preto/University of Sao Paulo (FMRP/USP). Mice were inoculated with *Aa* (ATCC 29522) cultured under anaerobic conditions and suspended in ∼100 μL of PBS solution supplemented with 2% CMC at 1×10^9 cfu placed in the oral cavity. At 48 h and 96 h, the inoculation was repeated. Negative controls received heat-killed sham bacteria or only PBS solution supplemented with 2% CMC. All protocols were approved by the local institutional animal care and use committees at the University of Pittsburgh and FMRP/USP.

For experiments that used *Pg* as a colonizing periodontopathogen in BALB/c mice, periodontitis was induced as described previously (3). Briefly, male BALB/c mice age 6 to 8 wk were purchased from Jackson Laboratories. To reduce the commensal oral bacteria, the drinking water of mice was modified with 15 mL/L of Sulfatrim pediatric suspension (sulfamethoxazole and trimethoprim, 2 mg/mL wt/vol and 0.4 mg/mL wt/vol; Henry Schein) for 10 d. After 10 d of antibiotic water, the mice were given clean drinking water for 5 d to prevent any direct microbicidal effects of the antibiotic solution on the colonization of the oral pathogen. Mice were then colonized three times during the first week at 2-d intervals with *Pg* (ATCC 33277) grown under anaerobic conditions. Bacteria were plated on *Brucella* blood agar supplemented with hemin and vitamin k1. On days of inoculation, *Pg* was suspended in BBL brain heart infusion (BHI) agar (BD Biosciences) supplemented with 2% CMC at 1×10^{11} cfu. Mice received 0.5 mL of the *Pg* BHI suspension orally administered with gavage feeding needle.

Canine. Nine female beagle dogs ∼12 mo of age were purchased from Marshall BioResources. Periodontal disease was induced as previously described (4). Briefly, all dogs received dental scaling and root planing on the mandibular fourth premolar and carnassial teeth 2 wk before ligature placement as a pretreatment to create a baseline for oral health. Every dog received twice-daily tooth brushing during the 2-wk pretreatment to maintain oral hygiene. To induce periodontitis, 2–0 silk sutures were placed at the cervix of the gingiva of the mandibular fourth premolar and carnassial teeth. Ligatures were held in place on the proximal and distal sides of each tooth by shallow notches made by using a round bur. Ligatures that had fallen off were replaced immediately, and a small amount of dental composite resin was used to ensure ligature maintenance. Microparticles were administered immediately after ligature placement and again 4 wk after ligature placement.

Murine Anti–Glucocorticoid-Induced TNF Receptor Treatment. Anti–glucocorticoid-induced TNF receptor (GITR; DTA-1) hybridomas were grown i.p. in mineral oil-injected nude mice as described previously (2). Briefly, the antibodies were purified from ascites by precipitation using ammonium sulfate (45% wt/vol), and subsequently purified by a G protein column (Amersham Biosciences) as described previously (2). A bicinchoninic method was used to quantify protein levels. The in vivo blockage of GITR molecules was performed by i.p. injection of 500 μg per

mouse of purified anti-GITR mAb diluted in PBS solution, performed 15 d after bacterial inoculation.

Assessment of Periodontal Disease. ***Mouse.*** To evaluate the extent of alveolar bone destruction, murine maxillary alveolar bone was quantified as described previously (2, 3). Briefly, resected maxillae were mechanically defleshed and exposed to Dispase or 3% hydrogen peroxide overnight to remove all soft tissue. Palatal and buccal faces of the molars were imaged using dissecting microscopes [Leica; or SZX10 with DP72 camera (Olympus)]. Digitized images were analyzed using ImageJ (National Institutes of Health) or ImageTool 2.0 (University of Texas Health Science Center). The area between the cementoenamel junction (CEJ) and the alveolar bone crest (ABC) was quantified by using arbitrary units of area or square micrometers.

Inflammatory cell infiltrate was analyzed from palatal periodontal lesions as described previously (2). Whole buccal and palatal tissues of maxillary molars were collected, weighed, and incubated in media (RPMI 1640, supplemented with $NaHCO_3$, penicillin/streptomycin/gentamicin, and 0.28 Wünsch units per milliliter of Liberase Blendzyme CI) for 1 h at 37 °C, with the dermal side down. Cell viability was assessed by trypan blue exclusion, and cell count was performed in a Neubauer chamber.

Total RNA extraction from periodontal tissues was preformed by using TRIzol reagent following the manufacturer's instructions (Life Technologies) as described previously (2). Briefly, cDNA was synthesized by using 3 μg of RNA through a reverse-transcription reaction (Superescript III; Invitrogen). Real-time PCR quantification of mRNA was preformed in a MiniOpticon system (BioRad) using SYBR Green PCR Master Mix (Applied Biosystems). For mRNA quantification, relative level of gene expression was calculated by using β-actin reference expression. The fold change was calculated as $2^{(-\Delta\Delta CT)} - 1$. Quantification of *Aa* in the palatal tissues was performed as previously described (5). Briefly, DNA was extracted from tissues by using a DNA purification system (Promega Biosciences) and quantified by using a MiniOpticon system, then normalized to tissue weight.

Measurement of proteins and cytokines from the periodontal tissues were performed as described previously (2). Briefly, protein was extracted from the palatal gingival after tissue homogenization in PBS solution. Samples were then centrifuged ($100 \times g$), and the supernatants were stored for testing (−70 °C). The concentrations of cytokines IL-10, TGF-β, TNF, cytotoxic T lymphocyte antigen 4 (CTLA-4), and RANKL were determined by ELISA kits (R&D Systems) and carried out according to manufacturer instructions.

Histological analysis of mouse periodontal tissues after *P. gingivalis* infection were performed as follows. All samples from *Pg*-infected mouse model were fixed in 10% formalin and embedded in paraffin. Sections were made at 6-μm thickness and stained with H&E. Forkhead box P3 (FOXP3)-positive cells were identified by immunohistochemistry using primary antibody against mouse FOXP3 (14-5773; eBiosciences), and SuperPicture 3rd Gen IHC Detection Kit (Life Technologies). Brightfield images were taken under a microscope (Eclipse TE2000-E; Nikon Instruments).

Canine. Pocket depth was measured at six points of each tooth, mesial and distal corner, and middle of both buccal and lingual sides. Bleeding on probing was recorded at each probing site. All clinical assessment was performed at 0, 4, and 8 wk after treatment. Gingival pocket depth is the distance between the cervixes of gingival to the attachment point of gingival epithelia to the tooth. The increase of pocket depth was calculated by subtracting the pocket depth at 0 wk from that at 4 wk or 8 wk by using the mean of all sites.

To quantify alveolar bone loss, dog mandibles were scanned in 70% ethanol by a vivaCT 40 microCT system (SCANCO). Three-dimensional images were reconstructed with SCANCO software at the same threshold across all samples. Each scan was reoriented with DataViewer (GE Healthcare). The images were reoriented by the planes adjusted to the CEJ, buccolingual center of the roots, and parallel to the root canal in the center of distal root of fourth premolar and mesial root of first molar. To assess the vertical bone reduction, the distance between CEJ and ABC was measured at the distal face and buccal face of the fourth premolar and the first molar after microCT image reorientation. Along the distal face of the tooth, the ABC–CEJ distance was measured at five points 0.3 mm apart, along the buccal face of the tooth. The ABC–CEJ distance was measured at 14 to 16 sites on premolar 4 and 26 to 28 sites on the molar spaced 0.6 mm apart from mesial to distal. To quantify an overall value of alveolar bone resorption per dog, we used a total summation of the linear CEJ–ABC distances over the (averaged left and right) premolars (19 sites per tooth) and molars (31 sites per tooth) of each dog. Bone loss was also represented as the average linear CEJ–ABC distance at the buccal and distal sites of premolar 4 and the first molar, averaged on a per-tooth basis.

Statistical Analyses. All data were confirmed to portray a normal distribution (determined by Shapiro–Wilk test) and further analyzed by using one-way ANOVA followed by Bonferroni or Tukey honestly significant difference post hoc test to compare differences between multiple groups. Student unpaired *t* test was used for all other statistical analyses. Differences were considered significant when $P < 0.05$. Statistics were performed by using GraphPad Prism or JMP Pro-10 software.

1. Jhunjhunwala S, et al. (2012) Bioinspired controlled release of CCL22 recruits regulatory T cells in vivo. *Adv Mater* 24(35):4735–4738.
2. Garlet GP, et al. (2010) Regulatory T cells attenuate experimental periodontitis progression in mice. *J Clin Periodontol* 37(7):591–600.
3. Yu JJ, et al. (2007) An essential role for IL-17 in preventing pathogen-initiated bone destruction: recruitment of neutrophils to inflamed bone requires IL-17 receptor-dependent signals. *Blood* 109(9):3794–3802.
4. Martuscelli G, Fiorellini JP, Crohin CC, Howell TH (2000) The effect of interleukin-11 on the progression of ligature-induced periodontal disease in the beagle dog. *J Periodontol* 71(4):573–578.
5. Garlet GP, et al. (2008) The essential role of IFN-gamma in the control of lethal Aggregatibacter actinomycetemcomitans infection in mice. *Microbes Infect* 10(5): 489–496.

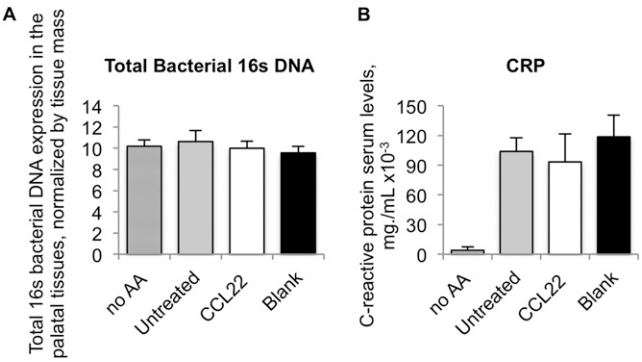

Fig. S1. CCL22 microparticle treatment does not alter overall oral bacteria numbers or serum levels of inflammatory marker C-reactive protein (CRP). Mouse periodontal tissues were collected 30 d after being colonized with *Aa* and treatment with CCL22 microparticles or unloaded polymer controls (blank) injected into the maxillary gingiva. Mice uninfected (no *Aa*) and mice infected but untreated served as controls. Microparticles were delivered on days −1, 10, and 20 relative to the first *Aa* inoculation. (*A*) Overall bacterial counts were quantified by measuring the 16s ribosomal DNA in the periodontal tissue of mice 30 d after initial inoculation, normalized by palatal tissue weight. No statistical differences were detected among the groups. (*B*) Serum collected postmortem 30 d after *Aa* colonization was analyzed for inflammatory marker CRP. Untreated, CCL22, and blank-treated mice had statistically higher levels of serum CRP than no-*Aa* uninfected controls. No differences were detectable among the infected animals.

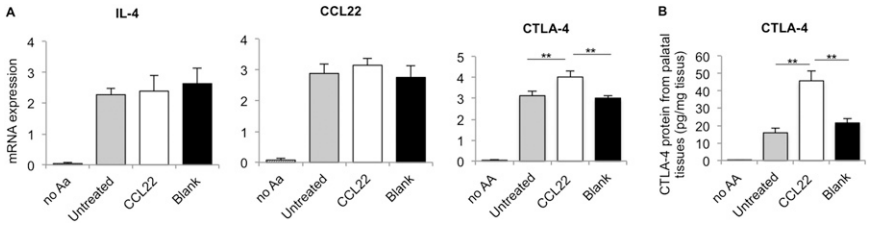

Fig. S2. CCL22 microparticle treatment leads to the up-regulation of cell suppressor molecule CTLA-4, but does not significantly alter endogenous CCL22 production or Th2 cytokine IL-4 levels. Expression of mRNA in periodontal tissue of mice 30 d after inoculation with *Aa* was analyzed by quantitative PCR. Expression was compared by the value of $2^{(-\Delta Ct)} - 1$ with reference to β-actin ($n = 5$ mice). Mice infected with *Aa* and given blank, unloaded microparticles or infected and untreated served as negative controls to CCL22 microparticle-treated mice, whereas mice not receiving *Aa* infection served as positive controls. Microparticles were injected into the maxillary gingiva on days −1, 10, and 20 relative to the first *Aa* inoculation. Statistics determined by one-way ANOVA followed by Bonferroni multiple-comparisons test (**significant at $P < 0.05$). Untreated, CCL22, and blank groups were statistically different from no *Aa*. (*A*) The mRNA expression of IL-4, endogenous CCL22 and CTLA-4, and CCL22 microparticle treatments had little effect on IL-4 and CCL22 levels. CTLA-4 mRNA expression was increased. (*B*) There was a significantly higher amount of CTLA-4 protein in the periodontal tissues.

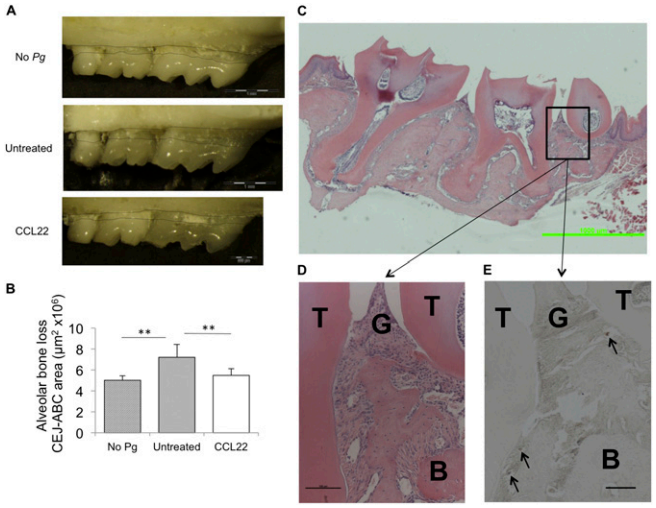

Fig. S3. CCL22 microparticles prevent alveolar bone resorption and recruit Tregs to the periodontium in a *P. gingivalis* experimental mouse model. BALB/c mice colonized with *Pg* treated with CCL22 microparticles injected in the maxillary gingiva at days −1, 20, and 40 showed significant reduction in alveolar bone loss and increased presence of Tregs in the periodontium 60 d after initial colonization. Uninfected mice (no *Pg*) and infected untreated mice served as controls for CCL22 microparticle-treated mice. (*A*) Representative microscope images of defleshed maxilla. (Scale bars: CCL22, 0.5 mm; untreated and no-*Pg*, 1 mm.) (*B*) Quantification of alveolar bone resorption represented by the area between the CEJ and ABC in square microns. (*C*–*E*) Representative immunohistochemistry images of CCL22 microparticle-treated mouse maxilla. Sections were stained with H&E (*C* and *D*), and immunohistochemistry was performed with anti-mouse FOXP3 antibody (*E*). FOXP3-positive cells were observed in periodontal tissue (arrows), $FOXP3^+$ cells were detectable only in sections from CCL22-treated mice. (Scale bars: *C*, 1,000 μm; *D* and *E*, 100 μm.) B, alveolar bone; G, gingiva; T, tooth (**$P < 0.05$ by ANOVA followed by Tukey HSD post hoc multiple-comparison test).

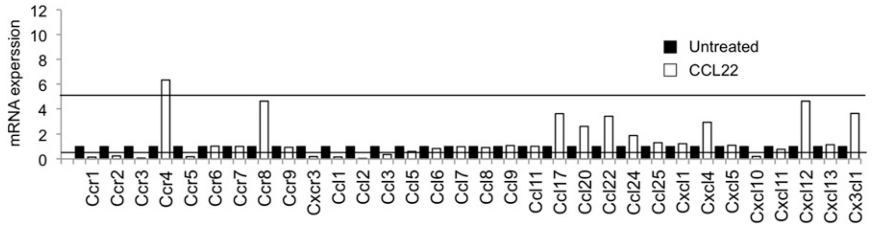

Fig. S4. CCL22 microparticle treatments alter chemokine and chemokine receptor expression. Expression of mRNA in the periodontal tissue of mice 30 d after *Aa* inoculation as analyzed with a custom PCR array. Samples were collected from periodontal tissue of mice that were infected with *Aa* without any particle treatment (untreated control, solid bars) and also infected with *Aa* and treated with CCL22-releasing particles (unfilled bars). Microparticles were injected into the maxillary gingiva on days −1, 10, and 20 relative to the first *Aa* inoculation. The threshold for up-regulation (more than fivefold increase) and down-regulation (less than 0.5 fold increase) in CCL22-treated group compared with the control group is indicated with lines.

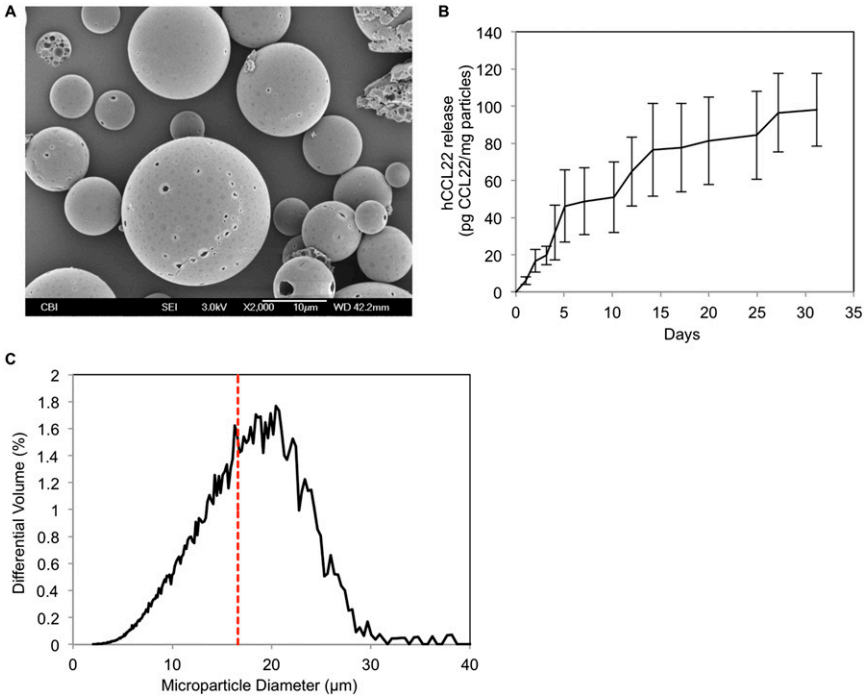

Fig. S5. Characterization of PLGA microparticles encapsulating rhCCL22. (*A*) Scanning EM image of PLGA microparticles encapsulating rhCCL22. (*B*) Cumulative fraction released from rhCCL22 microparticles determined by in vitro in PBS solution and measured by ELISA. (*C*) Volume impedance microparticle size distribution, with an average particle diameter of 16.6 μm represented by red dashed line (SD, ±5.8 μm).

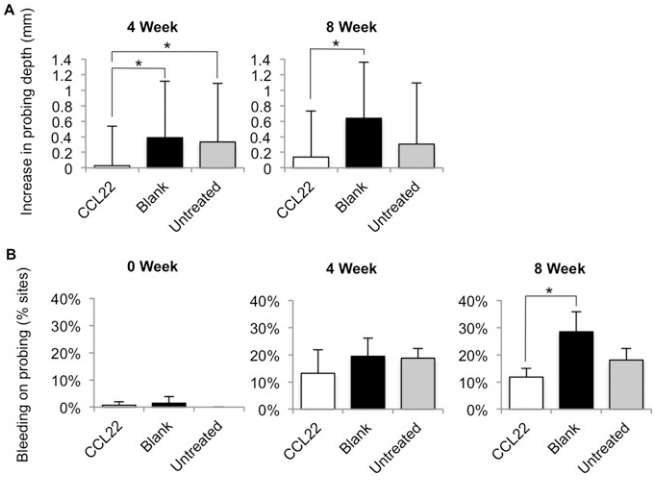

Fig. S6. CCL22 administration prevents exacerbation of clinical probing depths and bleeding upon probing scores in dogs. Beagle dogs receiving periodontal disease inducing ligatures at week 0 were monitored for pocket depth and bleeding on probing at six sites per tooth ($n = 3$ buccal, $n = 3$ lingual) of their second, third, and fourth premolars as well as their carnassial tooth. (*A*) Periodontal pocket depth increase as measured at 4 wk (vs. 0 wk) and 8 wk (vs. 0 wk) after treatment for molar sites. (*B*) The percentage of bleeding sites on probing of all of the probed sites at 0, 4, and 8 wk. Dogs treated with CCL22 microparticles deposited into the periodontal pocket at times 0 and 4 wk (CCL22) were compared with untreated and empty microparticle (blank) controls (*$P < 0.05$, Student t test).

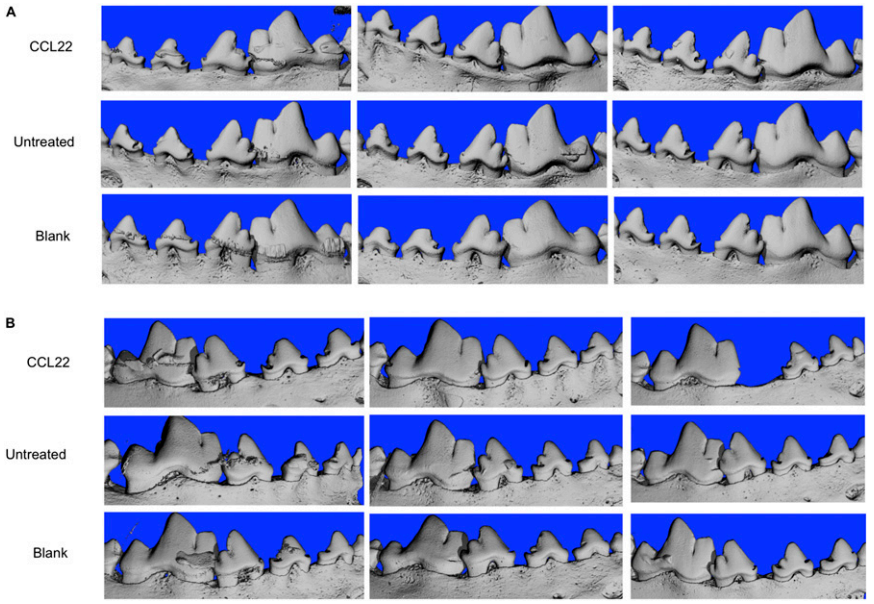

Fig. S7. Three-dimensional microCT images of the buccal face of the canine mandibular bone. (*A*) Representative 3D microCT images from mandibles of each animal on the buccal face of the left mandible postmortem, 8 wk of ligature placement, and (*B*) right mandible ($n = 3$ animals per group). One dog was missing premolar 4 at birth (*Top Right*), so no ligatures were placed on this tooth and it was excluded from the study. Dogs treated with CCL22 microparticles deposited into the periodontal pocket at times 0 and 4 wk were compared with untreated and empty microparticle (blank) controls.

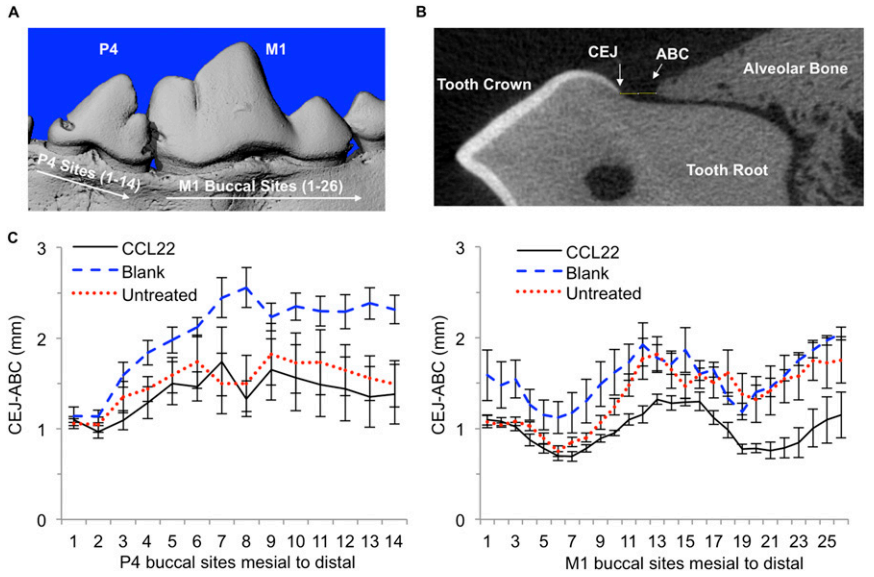

Fig. S8. MicroCT quantification of alveolar bone loss prevention in dogs treated with CCL22 microparticles. (*A*) Representative 3D X-ray microtomography image of beagle dog fourth premolar (P4) and first molar (M1). To quantify alveolar bone loss, five linear measurements of the distance between the CEJ and ABC were taken at 0.3-mm spacing on the distal face of the P4 and M1 (quantified and shown in Fig. 6). Additionally, the CEJ-to-ABC distance was measured at sites spaced 0.6 mm apart along the buccal face of the P4 (14 sites) and M1 (26 sites). (*B*) A representative image showing measurements of the CEJ to the ABC performed after reorientation of the microCT slices to align the apical roots and CEJ. (*C*) Linear bone loss between the CEJ and ABC along the buccal face of the left and right premolars (P4) and molars (M1) displaying the trends in alveolar bone resorption. Dogs treated with CCL22 microparticles deposited into the periodontal pocket at times 0 and 4 wk were compared with untreated and empty microparticle (blank) controls.