Supporting Information

Engineered Delivery of Dental Stem Cell-Derived Extracellular Vesicles for Periodontal Tissue Regeneration

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Experimental Procedure

Materials and Methods.

Chemicals and biologicals. Unless noted otherwise, all chemicals were purchased from Sigma-Aldrich, Inc. Glassware was acid-cleaned overnight and then thoroughly rinsed with Milli-Q water. Cell culture reagents, solutions, dishes, ELISA kits were obtained from Thermo Fisher Scientific, except as indicated otherwise.

Stem cells. Ten young (16-22 years old) healthy male and female individuals undergoing third molar extractions were chosen for extraction of gingival tissues (Institutional Review Board (IRB) approval from the University of California HS 07-00701). GMSCs were isolated and cultured according to previously published protocols [45]. Consequently, cells were pooled in equal numbers to compensate donor-dependent variability. Flow cytometric analysis was performed to confirm that cells were positive for MSC surface markers CD73, CD146, CD166, and Sca-1 and negative for hematopoietic cell markers CD31, CD34 and CD45 (BD Biosciences). Human BMSCs were purchased from Lonza. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco). BMSCs from passage 3-5 and GMSCs from passage 3-10 were used in this study.

sEV isolation. EV-depleted FBS was obtained by 18 h ultracentrifugation at 100,000 x g at 4 °C. Cells (50 - 80% confluent) were washed with PBS and cultured in medium containing EV-depleted FBS for 24 h. Cells were cultured in 300 cm^2 cell culture flasks (TPP Techno Plastic Products AG) with 40ml of media. For all EV isolations, cell viability was higher than 95%. Conditioned medium containing EVs was centrifuged at 2,000 x g for 10 min, filtered through a 0.22 μm filter and centrifuged at 100,000 x g for 3 h at 4 °C. sEV pellets were

resuspended in 100 µl PBS and stored at -80 °C until further use. One million of MSCs produced around 50 ug of sEV proteins in 24h while one million of GMSCs produced about 100 ug of sEVs as determined by Micro BCA protein assay (Thermo Scientific).

Preparation of polymeric microparticles. Polymeric microparticles were fabricated by dissolving carboxyl acid-terminated 50:50 (PURASORB PDLG 5004A) or 75:25 (PURASORB PDLG 7504A) poly DL-lactide/Glycolide (PLGA, Corbion Group) in dichloromethane (DCM) to create an organic phase (20 mg/ml). This was then poured into an aqueous phase of polyvinyl alcohol (PVA, 10 mg/ml) and homogenized at 10,000 rpm for 15 min. Deionized water at 40 °C was then added to the oil/water emulsion under magnetic stirring to evaporate the DCM. To prepare drug loaded microparticles, Minocycline (20 wt%/wt) was dissolved in DMSO and then added to the PLGA solution in DCM prior to formation of microparticles as mentioned above. Microparticles were centrifuged at 500 x g for 3 min, washed twice with deionized water, lyophilized, sterilized with x-ray irradiation, and finally stored at 4 °C until further use. Hydrodynamic diameter and surface charge of formed PLGA NPs was studied using dynamic light scattering (DLS) and zeta potential measurements (Zetasizer Nano, Malvern, UK). The average hydrodynamic size of the PLGA NPs microparticles was 8.4 ± 10.5 µm with a polydispersity index of 0.18 ± 0.06 and surface charge of 1.5 ± 0.6 mV.

sEV conjugation *via* peptides. Following microparticle formation, the GMSC sEVs were conjugated to the surface of the microparticles via MMP-sensitive peptides. 10 mg of microparticles were immersed in 500 µL of crosslinking solution containing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; 200 $mg.mL^{-1}$)/N-hydroxysuccinimide (NHS; 50 $mg.mL^{-1}$) in 2-(N-morpholino)ethanesulfonic acid (MES, pH 5.9) for 10 min and then washed with PBS. After the addition of the selected peptides (MMP in/-sensitive sequences), the microparticles were gently shaken overnight at 4 °C. Subsequently, microparticles were washed with NaCl (0.15 M; pH 8) for 30 min. The following peptide sequences were selected based on published reports [46] and were synthesized on solid resin using an automated peptide synthesizer according to standard F-moc chemistry.

MMP-sensitive sequence: GCRDGPQG↓IAGQDRCGC

MMP-insensitive sequence: GCRDGDQGIAGFDRCGC

The surfaces of GMSC sEVs (100 µg/ml) were also activated by sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate (Sulfo-LC-SPDP) crosslinker (150 µg/ml) to create

disulfide bonds to react with the thiol groups of cysteine residues on the MMP peptide conjugated to the surface of the microparticles (1 mg/ml). The reaction was conducted under gentle shaking overnight at 4 °C. Microparticles were then washed and kept at 4 °C prior to use. To quantify the sEVs loading on microparticles, 10 mg of EV-conjugated microparticles were dissolved in DMSO and then subjected to measurement using Micro BCA Protein Assay Kit (Thermo Fisher) that revealed that 1 mg of PLGA microparticles can load 21± 6.2 μg of GMSC sEVs.

sEV characterization

Western blot. Cells were lysed in Radioimmunoprecipitation assay (RIPA) buffer with protease inhibitors (HALT™ Protease Inhibitor Cocktail, EDTA-free [100X], Thermo Fisher Scientific) for 20 min on ice, and were then centrifuged for 15 min at 14,000 x g. The protein concentration was measured by Micro-BCA (Thermo Fisher Scientific) in the presence of 0.2% sodium dodecyl sulfate. 30 μg of sEV proteins or cell lysate were used for Western Blot analysis. For SDS-PAGE, samples were mixed with Laemmli Sample Buffer, boiled for 5 min at 95°C, and separated on 4-20% gradient polyacrylamide gels (Bio-Rad) before they were transferred to the PVDF membranes. The membranes were blocked with 5% non-fat milk for 1 h and were then incubated overnight at 4°C with primary antibodies: anti-CANX (1:500, rabbit polyclonal anti-human ABclonal); anti-CD63 (1:500, mouse monoclonal anti-human CD63 antibody, MEM-259, abcam); anti-FLOT1 (1:500, rabbit polyclonal anti-human ABclonal); anti-CD81 (1:300, mouse monoclonal anti-human CD81 antibody, Invitrogen); and anti-CD9 (1:500, mouse monoclonal anti-human CD9, clone MM2/57, Invitrogen). The membranes were then washed three times with 0.1% Tween in PBS for 5 min at RT, and were incubated with secondary antibody conjugated to horseradish peroxidase (donkey anti-rabbit IgG-HRP, sc-2313, or donkey anti-mouse IgG-HRP, sc-2314, Santa Cruz Biotechnology) for 2 h at 4°C. After the membranes were washed three times with 0.1% Tween in PBS, proteins were visualized with a chemiluminescence substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific).

Transmission electron microscopy (TEM). Undiluted suspensions of sEVs were applied on grids with a thin formvar/carbon film and were allowed to adsorb for 20 min. Excess liquid was blotted away with filter paper and the grids were then washed three times with 20 mM HEPES and 150 mM NaCl and negatively stained with 0.4% uranyl acetate and 3% methylcellulose for

1 min. Excess solution was blotted away, and the grids were air-dried. Samples were imaged using JEOL JEM 1200 EX operated at 120 kV.

Microfluidic resistive pulse sensing. The concentration and the size distribution of sEVs were analyzed by microfluidic resistive pulse sensing using a Spectradyne nCS1 (Spectradyne LLC) with a C-400/TS-400 cartridge.

sEV release study. The kinetics of sEV release dependent on MMP-2 was measured using a modified fluorescent quantification assay as previously reported [46-47]. Here, sEVs were first reacted with 1 mg/ml Fluorescein isothiocyanate (FITC) in PBS for 4 h and then subjected to filter purification to obtain FITC-sEVs. For the release study, 10 mg of FITC-EV conjugated microparticles were equilibrated at 37 °C in PBS overnight before adding 10 nM recombinant human MMP-2 (rhMMP-2) (R&D Systems) as reported previously [48]. The supernatant was collected after centrifugal precipitation of microparticles and replaced with PBS containing the same concentration of fresh 10 nM MMP-2 every day to preserve enzymatic activity. The collected supernatants were analyzed by quantification of the fluorescein signal (FITC-sEVs).

Minocycline release study. *In vitro* release of minocycline was studied by incubating 10^7 microparticles in 1 mL PBS (pH 7.4; supplemented with 10 mM $CaCl_2$) at 37 °C. At different time intervals, 500 µL of the supernatant was separated from microparticles, centrifuged, and replaced with an equivalent volume of fresh PBS solution. The concentration of released minocycline was determined by HPLC at 275 nm wavelength as reported previously [49].

Antibacterial assay. Kirby-Bauer agar disk diffusion assay was used to test antibacterial properties of PLGA-based microparticles against *Aggregatibacter actinomycetemcomitans* (*Aa*). Microparticles (200 µg) were suspended in deionized water and added to 6 mm diameter sterile paper disks. These paper disks were air-dried in a biosafety cabinet before use. A free drug control was prepared by adding equivalent amount of minocycline (10 µg) solution to paper disks. These disks were placed in agar plates inoculated with *Aa* at 37 °C. The inhibition zones were measured after 48 h.

T-cell isolation and activation. Five- to eight-week-old wild-type (C57BL/6) mice were purchased from the Jackson Laboratory and maintained in specific pathogen-free facilities at the University of California at Los Angeles (UCLA). All experiments on mice and cells

collected from mice were performed under a protocol approved by the Animal Research Committee and in accordance with UCLA's institutional policy on humane and ethical treatment of animals. Total T cells and CD4+ T cells were purified using magnetic-based, negative enrichment kits (Stem Cell Technologies)[50]. Cells were counted by hemocytometer using trypan blue exclusion (Calbiochem). T cells were cultured in RPMI supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, 1% sodium pyruvate, 1% HEPES buffer, and 0.1% μM beta-mercaptoethanol.

In vitro activation of purified T cells was done by culturing cells at a concentration of 1.5 × 10^6/mL in 24-well plate after treating the plates with anti-CD3 (2C11; Bio X Cell) at a concentration of 10 μg/mL followed by addition of 2 μg/mL soluble anti-CD28 (37.51; Bio X Cell). Soluble EVs (10 μg/mL) or microparticle-conjugated EVs (500 μg/mL) and 20 IU/mL of human IL-2 were added to the T cell media during the cell seeding. For flow cytometric analyses, antibodies against mouse CD4 (53-6.7), CD25 (PC61.5), CD44 (IM7), FoxP3 and CD16/CD32 ("Fc block") were purchased from BioLegend. Flow cytometry was performed on a Cytek DXP 10. FACS data were analyzed using FlowJo software (Tree star). Secretion of IL-10, IFN-g, and TNF-a cytokines in the non-diluted culture medium was assayed using an ELISA kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

For experiments to induce regulatory T cells (iTreg), CD4+ T cells were purified from mouse spleens by EasySep immunomagnetic negative selection (Stem Cell Technologies). Cells were then activated with anti-CD3 antibody (10 μg/mL)-coated plates supplemented with anti-CD28 antibody (2 μg/mL) in medium as mentioned previously. At the same time, EVs and IL-2 (20 IU/mL) with or without 1 ng/mL TGF-β were added to the media. After four days regulatory T cells were removed from wells and fixed, permeabilized, and stained with anti-CD25 and intracellular anti-Foxp3 antibodies for flow cytometry analysis.

Macrophage activation and differentiation. Monocytic THP-1 cells were maintained in culture in RPMI 1640 culture medium containing 10 % FBS supplemented with 10 mM HEPES, 1 mM pyruvate, 2.5 g/l D-glucose and 50 pM ß-mercaptoethanol. THP-1 monocytes were co-incubated with EVs (10 μg/mL) with or without 10 pg/ml of LPS for 24 h. Consequently, cells were washed with cold PBS, detached with EDTA (5 mM) and stained for flow cytometry. Cells were analyzed by flow cytometry, which was performed on a Cytek DXP 10. FACS data were analyzed using FlowJo software (Tree star). Cytokine secretion in the culture medium was assayed using an ELISA kit (Thermo Fisher Scientific) according to manufacturer's instructions.

THP-1 monocytes were differentiated into macrophages by 24 h incubation with 50 nM PMA followed by 48-72 h incubation in RPMI medium. Macrophages were polarized to M1 macrophages by incubation with 20 ng/ml of IFN-γ and 10 pg/ml of LPS for 24 h. Cell density of 10^6 in a 24-well plate was used in all monocyte/macrophage experiments.

Animal study. *In vivo* functionality of engineered microparticles was evaluated using a rat periodontal defect model, as described previously with some modifications [51]. Sixteen, two-month-old virgin male and female Sprague Dawley rats (Harlan Laboratories, Livermore, CA) were utilized for *in vivo* testing of the engineered periodontal treatments according to approved animal protocols. The animals were divided into four groups (four rats per group): (i) healthy control, (ii) no treatment (sham), (iii) blank PLGA microparticles without antibiotics and sEVs, (iv) PLGA microparticles loaded with minocycline, (v) minocycline and GMSC sEVs administered in solution (PBS), and (vi) engineered PLGA microparticles containing minocycline and GMSC sEVs. Briefly, mucoperiosteal flaps were elevated in rats in groups (ii-iv), uncovering the alveolar bone adjacent to the mesiolingual aspect of the first maxillary molars. The alveolar bone covering the root surfaces on the lingual side was removed with a dental bur under constant saline irrigation and then inoculated with *Aa* (10^9 cfu) in PBS cultured under anaerobic conditions. Three weeks later, 50 ul of microparticles (11 mg) or soluble controls (500 μg minocycline and 230 μg sEV) were injected into the defect site. Eight weeks post-implantation, the animals were sacrificed and the amounts of bone regeneration at defect sites were evaluated using μCT analysis. All the specimens were standardized, and μCT images were calibrated for proper comparative analysis. The vertical bone loss at each defect site was evaluated by measuring the distance between cementoenamel junction (CEJ) and alveolar bone crest as well as the relative alveolar bone area according to methods reported in the literature [51].

Characterization of inflammatory response at defect sites.

To estimate the inflammatory response kinetic, at different time intervals of 1, 2-, 4-, 6-, and 8-weeks post-treatment, 3-5 sterile paper points were placed around the inflamed sites for at least 30 seconds, and the concentrations of inflammatory cytokines (TNF-α) and anti-inflammatory cytokines (IL-10) were measured using ELISA kits (Thermo Fisher Scientific) according to the manufacturer's instructions. Moreover, populations of immune cells in periodontal tissue were analyzed as described previously [52] [53] [54]. Briefly, at 8 weeks post-implantation, buccal and palatal tissues of maxillary molars adjacent to the inflamed region were isolated and dissociated

using collagenase and DNase I (50 μg/mL) at 37 °C for 15 min. These enzymes were then inactivated with EDTA (20 μL/mL of solution). Tissues were then disaggregated and passed through a 0.7 μm cell strainer to obtain a single-cell suspension. Quantitative real-time PCR assays were used to analyze gene expression. RNA from isolated cells was isolated using Trizol reagent. RNA was reverse-transcribed and single-stranded cDNA was made using a Superscript III cDNA synthesis kit. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with normalization to the *Ct* of the housekeeping gene beta actin. Quantification of *Aa* in the periodontal tissues was performed as previously reported [52]. The concentrations of inflammatory cytokines (TNF-α) and anti-inflammatory cytokines (IL-10 and TGF-β) were measured after extraction of proteins from the gingival tissue using tissue homogenization using ELISA kits (Thermo Fisher Scientific).

Statistical analyses. The Kruskal-Wallis rank sum test, one-way ANOVA and two-tailed Student's t-test were utilized as appropriate to analyze the data at a significance of α or $p < 0.05$. Quantitative data are expressed as mean ± standard deviation (SD). To determine the number of specimens for the proposed experiments, power analysis was conducted based on our preliminary data.