Engineered Immunomodulatory Nanoparticles Inhibit Root Resorption and Ankylosis

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ABSTRACT

Introduction: External root resorption following avulsion injury is a complex process wherein differentiation of macrophages (Mϕ) to multinucleated osteoclasts is temporally regulated by resident periodontal fibroblasts (PDLF). The current study aims to assess the effect of engineered bioactive chitosan nanoparticles (CSNP), sustained released dexamethasone conjugated CSNP (CS-DEX) and CSNP functionalized with photosensitizer Rose Bengal (CSRB) for application in root resorption using an *in-vitro* PDLF-Mϕ direct coculture model and *in-vivo* delayed reimplantation model. **Methods:** PDLF-Mϕ direct coculture system was exposed to lipopolysaccharide (LPS), macrophage colony stimulating factor, receptor activator of nuclear factor kappa β ligand with or without CSNP/CS-DEX for 7 days. Clastic differentiation was assessed by tartrate resistant acid phosphatase (TRAP) staining on day 7. On day 2 and 7, immunofluorescence analysis was conducted to assess the expression of Mϕ polarization markers (CD80, CD206), multinucleation markers (NFATc1, STAT6) in Mϕ and matricellular protein periostin in PDLF and cytokine profiling in cell culture supernatants. Delayed replantation model with extraoral air dry/LPS exposure for 1h followed by root surface treatment with CS-DEX/CSRB was used in Wistar rats. After 21 days, rats were euthanized for histologic and immunofluorescence analysis. Statistical analysis one-way ANOVA with Tukey's multiple comparisons was used to analyze the data ($P < .05$). **Results:** CS-DEX significantly reduced $TRAP^+$ multinucleated cells and CSNP treatment showed no $TRAP^+$ cells. Immunofluorescence analysis showed that CSNP/CS-DEX reduced CD80, NFATc1 and STAT6 expression and increased periostin as expressed by fluorescence intensity. CSNP/CS-DEX significantly reduced TNFα, MMP9 and increased IL10, TGFβ1. Osteoprotegerin was upregulated only by CSNP. Root surface treatment in delayed replantation model showed that CS-DEX and CSRB substantially reduced the degree of resorption and ankylosis. Further, CD80, CD206, and MMP2 expression in groups with root surface treatment with CS-DEX and CSRB was lower than airdry/LPS group and similar to healthy control and NFATc1, STAT6, and MMP9 expressions were lower than healthy control. **Conclusion:** The engineered nanosized immunomodulatory bioactive materials chitosan nanoparticles functionalized with photosensitizer and dexamethasone effectively reduced the clastic differentiation of Mϕ in *in-vitro* coculture and minimized the resorption and ankylosis in a delayed reimplantation model. These biomaterials have the potential to serve as root modification agents, promoting favorable healing outcomes in cases of dental avulsion. *(J Endod 2024;50:1579–1592.)*

KEY WORDS

Root resorption; chitosan nanoparticles; immunomodulation; periodontal fibroblasts; macrophages

SIGNIFICANCE

Clinical Significance
Engineered sustained DEX releasing CS-DEX and extracellular matrix modifying CSRB effectively inhibited clastic differentiation of Mϕ in PDLF-Mϕ coculture and ankylosis in delayed replantation models via downregulation of CD80, NFATc1, STAT6, and MMP9 expression and upregulation of periostin, IL-10, and TGF-β1activity.

Root resorption (RR) in permanent dentition is a pathologic condition characterized by odontoblast-mediated degradation of dentin, cementum, or both[1]. Traumatic dental injuries in the age group between 2 and 20 years vary between 4.2% and 33.0%, with the highest incidence of 16% in maxillary permanent incisors[2,3]. The incidence of external inflammatory RR following traumatic dental injury, particularly in tooth avulsion, is 24.1%[2]. At the cellular level, RR is a complex multistep process regulated by the crosstalk

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between immune and resident cells under the influence of the local microenvironment. While macrophages (Mϕ) are the clastic precursor cells, their proliferation and clastic differentiation primarily depend on an array of growth factors and cytokines released by other immune cells (T lymphocytes), osteoblasts, and resident cells (periodontal fibroblasts (PDLF))[4,5]. PDLF-Mϕ direct coculture exposed to inflammatory and resorptive stimuli has shown to have a higher degree of clastic differentiation with higher levels of key mediators such as TNFα, IL1β, and MMP 9 compared to Mϕ monoculture[6]. PDLF from resorbing roots demonstrated higher receptor activator of nuclear factor kappa β ligand (RANKL) expression compared to nonresorbing roots[7]. Peripheral blood mononuclear monocytes grown in direct coculture with PDLF showed higher expression of genes related to clastogenesis when compared to monoculture[8]. Hence, modulating PDLF- Mϕ interaction can be a predictable therapeutic strategy to address RR.

Chitosan (CS) is the second most abundant natural biopolymer and is derived from chitin through deacetylation[9]. CS has recently gained prodigious attention because of its physicochemical and biological properties. CS structurally possesses rich free hydroxyl and amide groups that can be functionalized with carboxyl groups of bioactive molecules[10]. It can be synthesized into chitosan nanoparticles (CSNP), which offer distinctive characteristics because of their nanoscale size, increased reactivity, and large surface area to mass ratio[11]. CSNP are bioactive and biodegradable, and they have a wide range of biological activities, including antibacterial, antibiofilm, and anti-inflammatory properties[12,13]. A recent study demonstrated immunomodulatory effect of CSNP on the PDLF-Mϕ indirect coculture model by upregulating IL-10, TGF-β1, proteins with antioxidant activities, and by downregulating IL-1β, nitric oxide, and proteases[14,15].

CSNP conjugated with a photosensitizer, Rose Bengal (CSRB), has been shown to stabilize dentin extracellular matrix via surface infiltration and collagen crosslinking[10]. CSRB also inactivated proinflammatory mediators while promoting neo-tissue formation around Gram negative bacteria derived endotoxin lipopolysaccharide (LPS) contaminated dentin in an intraosseous implantation model[16]. A clinical case report showed successful healing of teeth with extensive root resorption when treated with low-level light activated CSRB[17]. CSNP functionalized with anti-inflammatory molecule, dexamethasone has been shown sustained release of DEX with positive effect on odontogenic differentiation of stem cells of apical papilla[18]. Stem cells of apical papilla treated with CS-DEX showed significantly higher ALP gene expression and calcified matrix deposition. The current study aims to assess the effect of engineered bioactive CSNP, sustained released dexamethasone conjugated CSNP (CS-DEX) and CSNP functionalized with photosensitizer (CSRB) for application in root resorption using an *in-vitro* PDLF-Mϕ direct coculture model, and *in-vivo* delayed reimplantation model.

MATERIALS AND METHODS

Materials

The study was approved by the Ethics Review Board of the University of Toronto (*In-vitro*: # 40166, *In-vivo*: #20012802). Engineered nanoparticles used in the present study were previously synthesized and characterized[18,19]. The concentration of CSNP/CSRB (0.1 mg/mL) and CS-DEX (30 μg/mL) was chosen based on the preliminary experiments set based on a previous study[15,18].

In-vitro Cell Culture Experiments

Human acute monocytic leukemia cells (THP-1 monocytes ATCC TIB-202 TM -Rockville, MD, USA) were maintained in complete RPMI 1640 (#R8758, Sigma Aldrich, St. Louis, USA) with 10% heat-inactivated fetal bovine serum (FBS-# 19C448, Sigma Aldrich, USA), 1% antibiotic/antimycotic (#15240-062, Gibco, USA), and 0.1% β mercaptoethanol (#21985023, Gibco, New York, USA) at 37°C in a humidified atmosphere containing 5% CO2. THP-1 cells (2.5 × 105 cells/mL) were differentiated to Mϕ using 60 mg/mL phorbol 12-myristate-13-acetate (PMA-Sigma Aldrich, St. Louis, USA) as previously described[15]. Human PDLF were provided as a kind gift from Dr. Douglas Hamilton's lab, Schulich's School of Medicine & Dentistry, London, ON, Canada. PDLF were cultured in complete DMEM (D5796-Sigma-Aldrich, Canada) supplemented with 10% heat-inactivated FBS, 1% antibiotic/antimycotic at 37°C incubator with 5% CO_2 and humidified atmosphere. Cells from the 3rd–5th passage were used, and experiments were performed in triplicates.

PDLF-Mϕ direct coculture (Supplemental Figure S1A) was exposed to one of the following stimuli: inflammatory [1 μg/mL *Porphyromonas gingivalis* lipopolysaccharide (LPS)][20] and resorptive [30 ng/mL RANKL[21] (AF-310-01, PeproTech, NJ, United States) and 25 ng/mL macrophage colony stimulating factor[21] (AF-300-25, PeproTech, NJ, United States)] or combination of inflammatory and resorptive stimuli with or without NPs (CSNP: 0.1 mg/mL or CS-DEX: 30 μg/mL) for 7 days at 37°C in a humidified atmosphere containing 5% CO_2. Cells received fresh media with respective stimuli on day 3. After 7 days, cells were stained with commercially available TRAP stain. At the end of day 2 and 7, immunofluorescence staining was done to assess the expression of Mϕ markers (CD80 and CD 206), multinucleation transcription factors (NFATc1 and STAT6) and periostin.

Tartrate Resistant Acid Phosphatase (TRAP) Staining

Cells were fixed with 10% neutral buffered formalin for 10 min at room temperature after a gentle wash with PBS. Cells were permeabilized with 0.1% Triton X-100 for 5 min at room temperature, washed with PBS, and air dried for 15 min. TRAP solution was prepared using a leukocyte acid phosphatase TRAP kit (386A-Sigma Aldrich, St. Louis, USA) as per the manufacturer's instructions. The[6] cells were treated with TRAP staining solution for 1h at 37 °C in the dark, followed by PBS wash to remove the unbound stain. Nuclei were counterstained with acid hematoxylin at 37 °C for 5 min. Cells were washed, and images were captured using Carl Zeiss Microscopy GnbH (37081, Gottingen, Germany). TRAP activity was observed as purplish to dark red granules in the cytoplasm. Nine microscopic fields were selected from each sample, and a total number of TRAP-positive multinucleated cells ($TRAP^+$ MNC) was counted.

Immunofluorescence Analysis

Cells were prepared for immunofluorescence analysis by treatment with 10% neutral buffered formalin for 30 min, permeabilizer 0.1% Triton X-100 for 5 min, and SeaBlock serum free-PBS (ab166955, Abcam Inc, USA) for 1 hour with three-time PBS wash between each step. Cells without further wash were treated with fluorophore-conjugated antibodies diluted in a SeaBlock serum free-PBS. Details of the stains and the dilutions are shown in Supplemental Table S1. Cells were incubated at 4 °C overnight in the dark, washed thrice, followed by counterstaining with nuclear stain 40, 6-diamidino-2-phenylindole for 5 min. Cells were washed to remove residual stains, and images were captured using a confocal laser scanning microscope (Leica SP8 Lightning Confocal/Light Sheet, Leica Microsystems, Richmond, IL, USA). Experiments were conducted in triplicates and repeated thrice ($n = 3$). In each cell culture well, 6 regions were randomly chosen for representative images and used for statistical analysis. The fluorescence intensity of each marker was assessed based on the intensity sum normalized to relative area in the

region of interest using Imaris software (Imaris 9.9; Oxford Instruments, Switzerland)[6].

Cytokine Profiling

Cell culture supernatant collected on day 2 and 7 was centrifuged at 10,000 × g for 5 minutes and frozen at - 80 °C until analysis. A multiplex immunoassay for analysis of inflammatory cytokines [tumor necrosis factor-alpha (TNF-α), interleukins (IL-6, IL-10)] and proteases [MMP2 and MMP9] was performed using the HCYTOMAG-60K-05 HCYTOMAG-60K and HMMP2MAG-55K-02 Milliplex MAP (Millipore, Billerica, MA, USA) respectively. Osteoprotegerin (OPG) and transforming growth factor-β1 (TGF-β1) levels were analyzed using the ELISA kits RAB0484-1 KT and RAB0460-1 KT (Millipore Sigma, Oakville, ON, Canada), respectively.

Gene Expression of Osteocalcin (OCN) and Type I Collagen (COL1)

PDLF monoculture was treated with DMEM with or without CSNP: 0.1 mg/mL or CS-DEX: 30 μg/mL for 7 days, and gene expression of osteocalcin and COL1 was assessed. Primer sequences used are tabulated in Supplemental Table S2. Total RNA was extracted using a RNeasy kit (Qiagen Inc, Hilden, Germany) per the manufacturer's protocol. QiaShredder columns were used to collect the RNA, and the concentration of RNA was evaluated spectrophotometrically (Synergy H4 Hybrid Multi-mode Microplate Reader; BioTek, Winooski, VT). The cDNA was synthesized using the SensiFAST cDNA Synthesis Kit per the manufacturer's protocol. Real-time reverse-transcription polymerase chain reaction was run as follows: 95 °C for 15 minutes as the denaturation program, followed by 40 amplification cycles (95 °C for 15 sec, 58 °C for 30 sec and the final temperature increased to 95 °C for 30 seconds to ensure the specificity of the reaction. The gene expression osteocalcin (OCN) and Type I collagen (COL1) were normalized to the expression of the housekeeping gene GAPDH.

In-vivo Study: Delayed Replantation Model

Fifty four male Wistar rats of 6-8 weeks of age and weighing 150-200 g were selected for the delayed replantation model (Supplemental Figure S1B). Rats were provided with a standard diet prior to experiments and a soft diet on the first one-week postoperative. Following adequate anesthesia with Ketamine hydrochloride 75-90 mg/kg and Xylazine 5-10 mg/kg body weight, the maxillary right incisor was luxated using a winged Luxator Elevator 1.5 mm - Short Handle Elevator (Dr. Brett's Pets, Lake Mary, FL. USA) and extracted using curved extraction forceps. Extracted tooth from each rat was assigned to extra oral Airdry[21,22], or LPS (1 μg/mL)[20], or PBS exposure for 1h followed by with or without root surface treatment with CS-DEX, CSRB or CS-DEX + CSRB for one min (Supplemental Figure S1B). Concentration of CS-DEX and CSRB used for root surface treatment was 30 μg/mL and 0.1 mg/mL respectively. For the combination group, 30 μg of CS-DEX and CS-RB was topped up to final total concentration of 0.1 mg/mL. For groups with CSRB, light (540 nm) activation to a total dose of 40 J/cm^2 in 1 min was done. A sample of 6 rats in each group was assigned based on the literature[21], and the maxillary left incisor in each rat served as their healthy control. Rats received Buprenorphine SR (1-1.2 mg/kg body weight) subcutaneously for 2 days to reduce any pain. Rats were euthanized after 21 days with a CO_2 chamber, and maxillectomy was performed to separate the maxillary incisors with surrounding periodontium alveolar bone from the remaining parts of the maxilla. Samples were fixed with 10% neutral buffered formalin for 24 h followed by decalcification using 12.5% EDTA. Samples were then serially dehydrated with 75%, 95%, and 100% ethanol and xylene treatment to embed them in paraffin wax. and 5 μm thickness sections were prepared perpendicular to the long axis of the tooth from the paraffin tissue block, sequentially rehydrated and subjected to histologic (hematoxylin and eosin)[16] and immunofluorescence staining[6]. Details of the stains and the dilutions are shown in Supplemental Table S1. Slides were scanned with Zeiss Axio Scan.Z1 (Carl Zeiss, Oberkochen, Germany) at × 40 objective. Fluorescence intensity for stain was measured and normalized to the area using Zeiss Zen 3.7 (blue edition).

Statistical Analysis

All *in-vitro* experiments were performed in triplicates, and data obtained are presented as the mean ± standard deviation (SD). GraphPad Prism 8 software (GraphPad Prism 8.0.1; GraphPad, La Jolla, CA) used one-way ANOVA with Tukey's multiple comparisons to analyze the data. P value $< .05$ was considered statistically significant.

RESULTS

TRAP + Multinucleated Cells (MNC)

PDLF-Mϕ coculture treated with CSNP showed a complete absence of TRAP$^+$ MNC, and CS-DEX showed a significant reduction in the TRAP$^+$ MNC compared to the control without CSNP/CS-DEX (Fig. 1*A* and *B*). Based on the TRAP staining results, it can be suggested that CSNP demonstrated higher inhibitory effect on clastic differentiation of Mϕ than CS-DEX.

Cytokine Profiling

PDLF-Mϕ direct coculture treated with CSNP and CS-DEX treatment showed significant upregulation of anti-inflammatory cytokines IL10 (Fig. 1*C*) and TGF-β1 (Fig. 1*D*). Among the experimental stimuli, inflammatory and combination group treated with CS-DEX showed greater IL10 levels than in CSNP (Fig. 1*C*). NP-mediated upregulation of IL10 levels was higher on day 2 than on day 7. While both CS-DEX and CSNP upregulated TGFβ1, higher levels were observed in resorptive, and combination group treated with CS-DEX than CSNP at day 2. At day 7, overall higher levels were observed in CSNP than CS-DEX (Fig. 1*D*). Increase in IL6 (Fig. 1*E*) and MMP2 (Fig. 1*H*.) was observed with both NP with greater upregulation by CS-DEX than CSNP. Pro-inflammatory cytokine TNFα (Fig. 1*F*) and clastic specific protease MMP9 (Fig. 1*G*) was significantly reduced by both NP with greater downregulation by CSNP than CS-DEX. At day 2, CSNP showed more significant reduction of TNFα in inflammatory and combination groups, and CS-DEX showed more significant reduction in the resorptive group. At day 7, both CSNP and CS-DEX showed similar levels of TNFα reduction. MMP9 was significantly reduced by both CS-DEX and CSNP, with a higher reduction with CSNP at both time points (Fig. 1*G*). MMP2, in contrast, was upregulated linearly from day 2 to 7. CS-DEX-treated groups showed a more significant increase in MMP2 than CSNP (Fig. 1*H*). OPG, also known as osteoclastogenesis inhibitory factor, was upregulated only in CSNP treated groups. OPG was increased at both time points with CSNP treatment, and the highest levels were observed in the inflammatory group, followed by the combination and then the resorptive group (Fig. 1*I*).

Immunofluorescence Analysis

PDLF-Mϕ direct coculture treated with CSNP and CS-DEX showed lower intensity of CD80 expression on day 2 and 7 with the highest reduction in inflammatory and combination groups treated with CS-DEX (Fig. 2*A* and *C*). Fluorescent intensity of CD206 expression was not upregulated in both CSNP and CS-DEX treated groups (Fig. 2*A* and *D*). Both CSNP and CS-DEX reduced NFATc1 intensity to a similar level at both time points (Fig. 2*B* and *E*). STAT6 expression was substantially

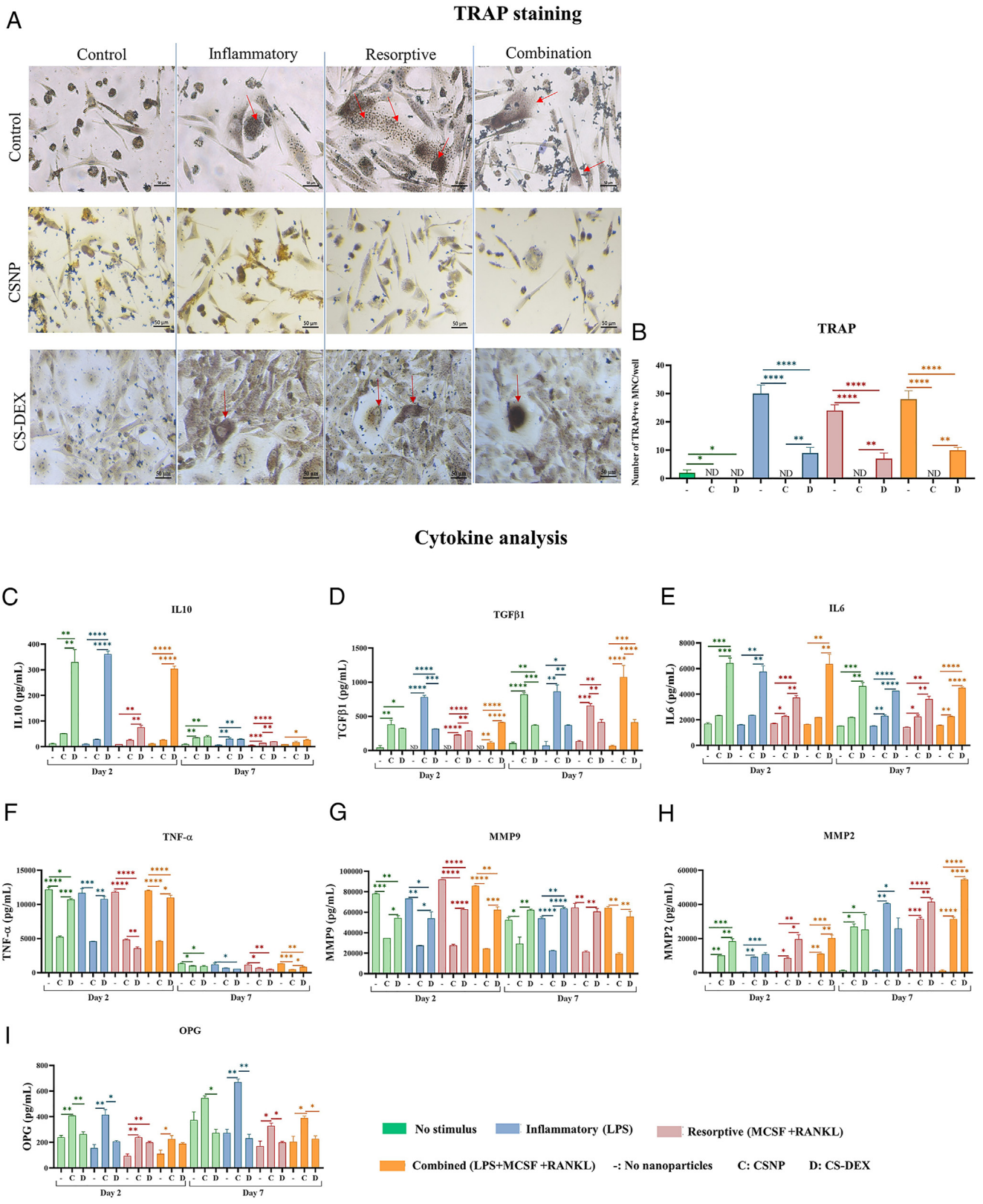

FIGURE 1 – Representative images of TRAP staining of PDLF-Mϕ coculture exposed to inflammatory, resorptive, and combination stimuli with and without nanoparticles (NPs) (*A*) and quantitative data of TRAP results (*B*). Cytokines analysis of supernatant collected on day 2 and 7 from PDLF-Mϕ coculture exposed to inflammatory, resorptive, and combination stimuli with and without NPs. Data were analyzed with one way ANOVA test with Tukey multiple comparison was performed using GraphPad prism V8.01. *: $P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$. TRAP, Tartrate resistant acid phosphatase; ND, Not detected; -: No nanoparticles, C:CSNP, D:CS-DEX, LPS, Lipopolysacharide; MCSF, Macrophage colony stimulating factor; RANKL, Receptor activator of nuclear factor kappa beta. Red arrows in Figure 1*A* indicate TRAP + multinucleated cells.

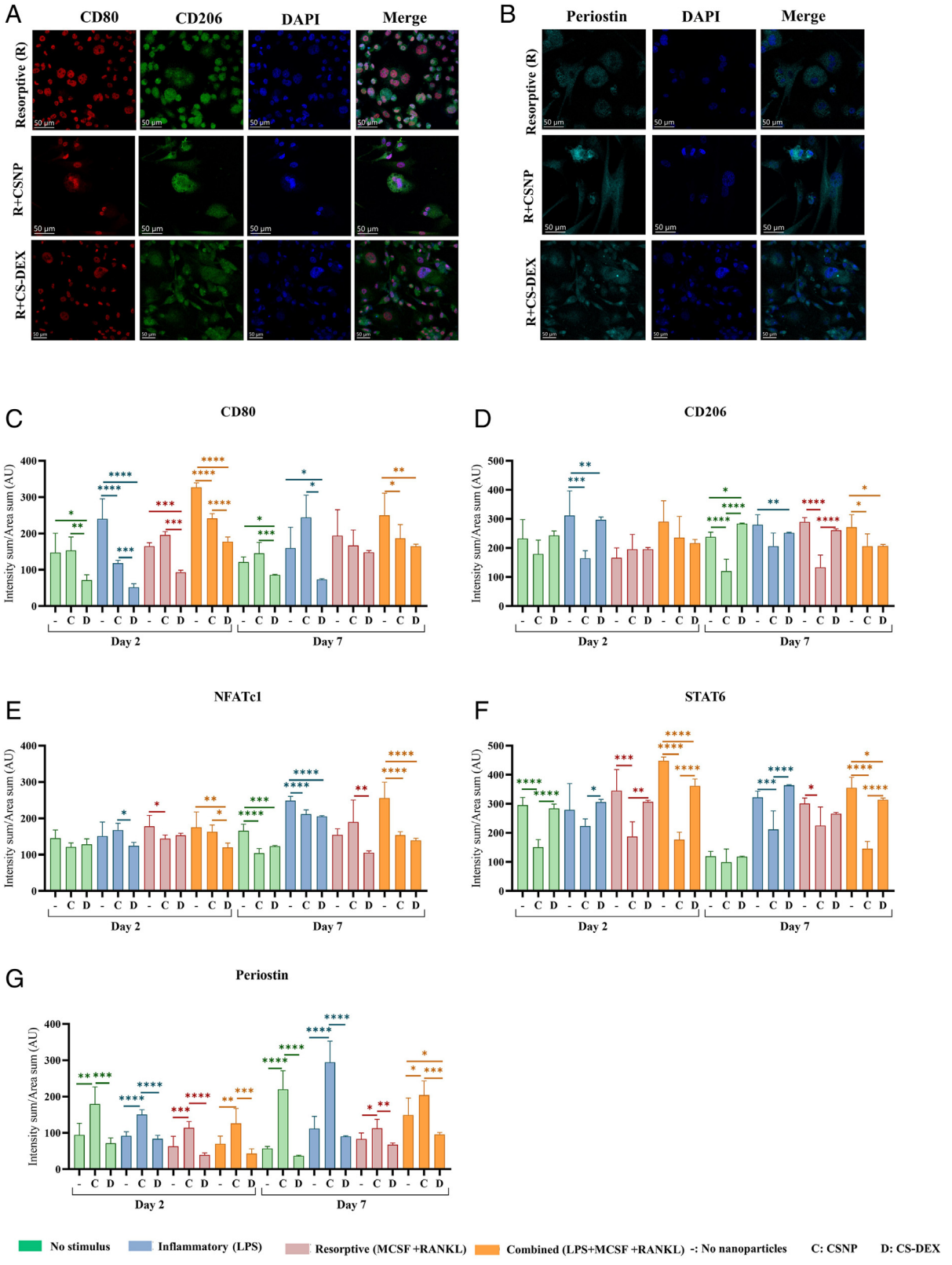

FIGURE 2 – Representative immunofluorescence images of Mϕ markers-CD80, CD206 (*A*), and Periostin (*B*) from day 7 interaction from resorptive group with and without CSNP (0.1 mg/mL)/CS-DEX (30 µg/mL) treatment. (*D–G*) shows quantification of CD80 (*C*), CD206 (*D*), NFATc1 (*E*), STAT6 (*F*), and Periostin (*G*) expressions. Data were analyzed with one way ANOVA test with Tukey multiple comparison was performed using GraphPad prism V8.01. Difference with $P < .05$ was considered as statistically significant. *: $P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$. No nanoparticles, C:CSNP, D:CS-DEX, LPS, Lipopolysaccharide; MCSF, Macrophage colony stimulating factor; RANKL, Receptor activator of nuclear factor kappa beta.

downregulated in resorptive, and combination groups treated with CSNP rather than CS-DEX (Fig. 2*C* and *F*). Periostin expression was upregulated only by CSNP, with the highest levels observed in inflammatory, followed by combination and resorptive groups (Fig. 2*C* and *G*).

Gene Expression of OCN and COL1

PDLF monoculture treated with CSNP and CS-DEX for 7 days showed different levels of gene expressions of OCN and COL1 (Supplemental Figure S2). While both CSNP and CS-DEX showed higher fold change in OCN levels than the control, OCN levels in PDLF treated with CS-DEX were significantly higher than PDLF treated with CSNP. With respect to COL1, significantly higher levels were observed in PDLF treated with CSNP than PDLF fromcontrol and CS-DEX group. CS-DEX and control did not show a difference in the COL1 levels. OCN: COL1 ratio for CSNP and CS-DEX was 0.45 and 2.97, respectively.

Animal Study: Histologic Analysis

Tissue response to dental avulsion can be seen as inflammatory resorption or ankylosis depending on the severity of tissue damage, loss of viable PDLF and presence of bacterial stimuli[2,23]. Degree of root resorption and ankylosis in air dry, LPS exposure with and without root surface treatment with nanoparticles was assessed by staining the tissue sections with hematoxylin and eosin staining. Stained sections were divided into 4 quadrants (Fig. 3*A*.), and each quadrant was assessed for the presence of resorption and ankylosis. Resorption was classified into grades (Fig. 3*A*) based on the depth of involvement[24], and the percentage and area of resorption for each grade and percentage and root surface perimeter with ankylosis were calculated for each quadrant and compiled to represent the data entire root surface (Fig. 3 *B*–*G*). Root surface treatment with NPs showed a lower percentage of resorption compared to airdry and LPS (Fig. 3*D* and *E*). NPs treated groups showed significantly lower areas of resorption particularly in Grades III-V (Fig. 3*D* and *C*). Highest percentage and root perimeter with ankylosis was observed in airdry (Fig. 3*E* and *F*) and NPs groups showed significant reduction in the degree of ankylosis. Samples from LPS + CS-RB, Airdry + CS-DEX + CS-RB, and LPS + CS-DEX + CSRB groups showed zero ankylosis.

Immunofluorescence Analysis

Highest expressions of CD80, CD206, NFATc1, STAT6, MMP2, and MMP9 were observed in LPS than in Airdry and significantly lower in all the NPs treated groups (Fig. 3*H*–*P*). CD80, CD206, NFATc1, and STAT6 expression in Airdry + CS-DEX and LPS + CS-DEX were similar to healthy control (Fig. 3*I*–*L*). CS-DEX + CS-RB combination groups showed CD80, CD206, NFATc1, and STAT6 expressions significantly lower than healthy control (Fig. 3*I*–*L*). MMP2 levels (Fig. 3*M*) in all NP-treated groups except LPS + CS-DEX + CS-RB were similar to healthy control. MMP9 was significantly reduced in all NP-treated groups (Fig. 3*N*).

DISCUSSION

Differentiation of Mϕ to multinucleated odontoclasts followed by degradation of immunologically vulnerable dentin is a complex process temporally regulated by cell surface and secreted proteins expressed by resident PDLF and other immune cells. In *in-vitro* model, bacterial LPS of *P. gingivalis*, a proinflammatory glycolipid[25], was used to simulate an inflammatory microenvironment, and macrophage colony stimulating factor and RANKL were used to simulate a resorptive microenvironment[26]. Engineered bioactive nanoparticles were used in the current study to investigate its therapeutic potential on oclastic differentiation of Mϕ in a PDLF-Mϕ direct coculture system exposed to different resorptive stimuli. In *in-vivo* experiment, delayed replantation model with extra oral dry and LPS exposure for used to simulate dental avulsion. Extra oral dry period was used as it simulates noxious stimuli that negatively affects PDLF viability and initiate ankylosis[21,22]. *P. gingivalis* derived LPS is a potent endotoxin that stimulate proinflammatory mediators from PDLF and macrophages favoring clastic differentiation of clastic precursors and hence LPS was used to simulate bacterial derived inflammatory stimuli[20]. Extra-oral dry or LPS exposure was maintained for 1 hour as it has been shown to negatively affect the prognosis[21].

Chitosan nanoparticles over conventional powder-based root surface agents offer several advantages in dental avulsion. With their cationic nature, CSNP and CSNP functionalized with bioactive molecules undergo electrostatic interaction with negatively charged cell membranes of mammalian cells resulting in rapid cellular uptake of via macropinocytosis, clathrin-mediated endocytosis, and phagocytosis[15] and would then participate in cellular functions that regulate various biological activities. Free hydroxyl and amide groups in the chitosan offers possibilities to functionalise the CSNP with bioactive molecules. In the vitro experiments, CSNP and CSNP functionalised with anti-inflammatory agent dexamethasone used to modulate PDLF-Mϕ interaction in inflammatory resorptive environments. For experiments in the animal study, apart from CS-DEX, CSNP functionalized with photosensitizer Rose Bengal dye owing to its positive effects collagen crosslinking and dentin stabilization was used[27].

Osteoclast formation starts with mononuclear osteoclastic precursor passing through a series of cellular stages that include a) prefusion polarization of the cells, b) chemotaxis, cellular adhesion and cytoskeletal rearrangements, c) membrane fusion and multinucleation, and d) postfusion macrophage expression of cytosolic enzymes[25]. Multiple activated macrophages then coalesce to form multinucleated osteoclasts with sophisticated hard tissue degradation proteases and cytokines expression[25,28]. While NFATc1 is considered as the master transcription factor of multinucleated clastic cells, recent studies have shown positive role of STAT6 in multinucleation[6,25]. Hence, both NFATc1 and STAT6 were used in the study as a potential outcome measure of clastic differentiation of Mϕ. Multinucleated clastic cells secrete cytoskeletal marker TRAP that eliminates endocytosed material[26].

CSNP treated PDLF-Mϕ coculture showed a complete absence of $TRAP^+$ MNC. In contrast, PDLF-Mϕ coculture with no CS-DEX showed a significantly lower number of $TRAP^+$ MNC in all experimental stimuli groups than control without NPs. While both CSNP and CS-DEX were able to reduce the clastic differentiation of Mϕ in PDLF-Mϕ direct coculture, they demonstrated distinct difference in their mechanism of action as shown in cytokine profiling and immunofluorescence analysis. Although both CSNP and CS-DEX significantly increased anti-inflammatory cytokines IL10 and TGFβ1, nanoparticles showed a temporal difference in their activity. IL10 was significantly upregulated in the early phase while TGFβ1 in late phase. Between the nanoparticles, greater effect on TGFβ1 and IL10 was observed with CSNP and CS-DEX respectively. CSNP showed higher downregulation of proinflammatory cytokine TNFα and clastic specific protease MMP9 and higher upregulation of osteoclast inhibitory factor OPG than CS-DEX thus explaining greater reduction in TRAP activity.

Immunofluorescence analysis showed that NPs were able to effectively downregulate Mϕ polarization marker CD80 and multinucleation markers NFATc1 and STAT6 in both *in-vitro* and animal study. These positive effects particularly in the animal study were greatest when CS-DEX and CSRB were used

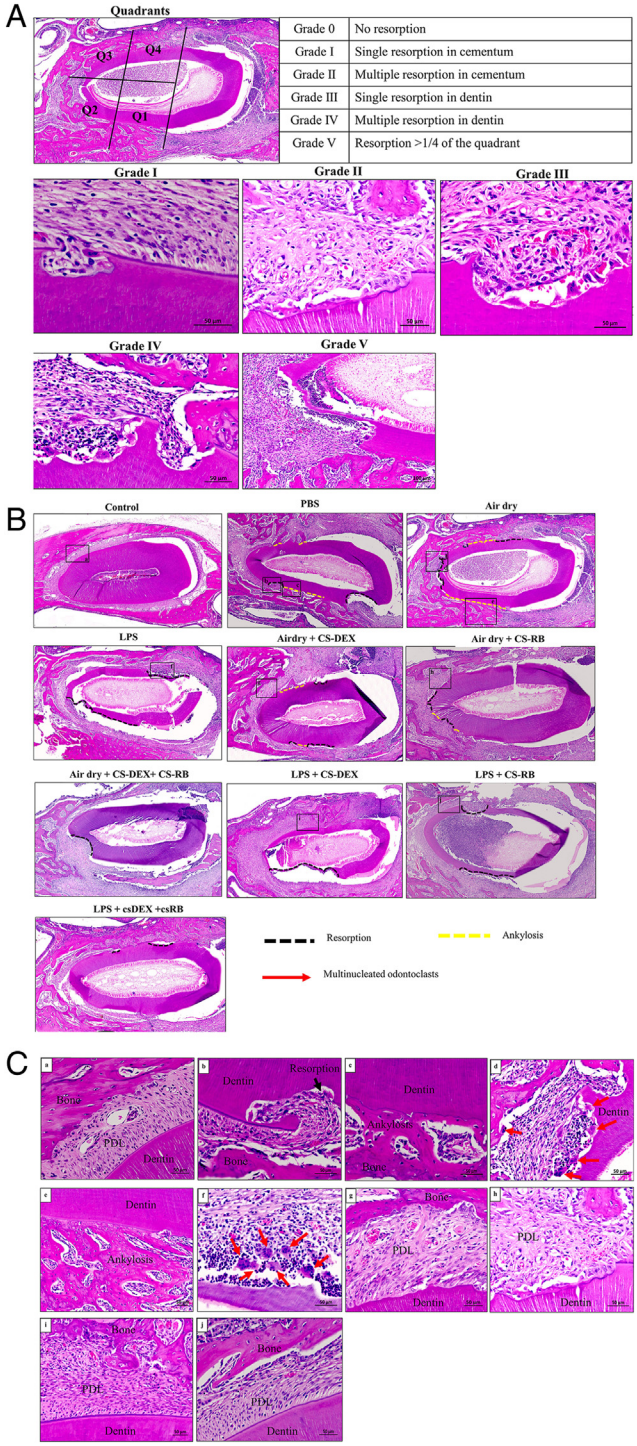

FIGURE 3 – (*A*) Hematoxylin and eosin-stained sections were divided into 4 quadrants and each quadrant was assessed for presence of resorption and ankylosis. Resorption was graded as Grade I to Grade V depending on the severity. Figure 3*B* and *C*: (*B*) shows lower magnification images of hematoxylin and eosin-stained sections from different groups. (*C*) shows higher magnification of rectangular shaped regions of interests from the lower magnification images B. Figure 3*D*–*G*: (*D*) and (*E*) show data of percentage and area of resorptions in different groups. (*F*) and (*G*) show data of percentage and perimeter of root surface with ankylosis. Area of resorption between the groups and perimeter of root surface with ankylosis between the groups was compared with one way ANOVA test with Tukey multiple comparison using GraphPad prism V8.01. Difference with $P < .05$ was considered as statistically significant. Different alphabets indicates the differences were statistically significant. A, Air dry; L, LPS; D:CS-DEX, R: CSRB. Figure 3*H*: Immunofluorescence images of NFATc1 and STAT6 expression of control, PBS, Airdry, and LPS at lower magnification (Scale bar 200 μm). Higher magnifications of regions of interests are shown as lower alphabets a-h. A, Air dry; L: LPS, D:CS-DEX, R: CSRB. Figure 3*I*: Immunofluorescence images of NFATc1 and STAT6 expression of Airdry + CS-DEX, Airdry + CS-RB, Airdry + CS-DEX + CS-RB at lower magnification (Scale bar 200 μm). Higher magnifications of regions of interests are shown as lower alphabets i-n. A: Air dry, L: LPS, D:CS-DEX, R: CSRB. Figure 3*J*: Immunofluorescence images of NFATc1 and STAT6 expression of LPS + CS-DEX, LPS + CS-RB, LPS + CS-DEX + CS-RB at lower magnification (Scale bar 200 μm). Higher magnifications of regions of interests are shown as lower alphabets o-t. A: Air dry, L: LPS, D:CS-DEX, R: CSRB. Figure 3*K*–*P*: Quantitative data of immunofluorescence analysis of CD80, CD206, NFATc1, STAT6, MMP2 and MMP9 expression. One way ANOVA test with Tukey multiple comparison was performed using GraphPad prism V8.01. Different alphabets indicate the differences in the comparison are statistically significant ($P < .05$). A: Air dry, L: LPS, D:CS-DEX, R: CSRB.

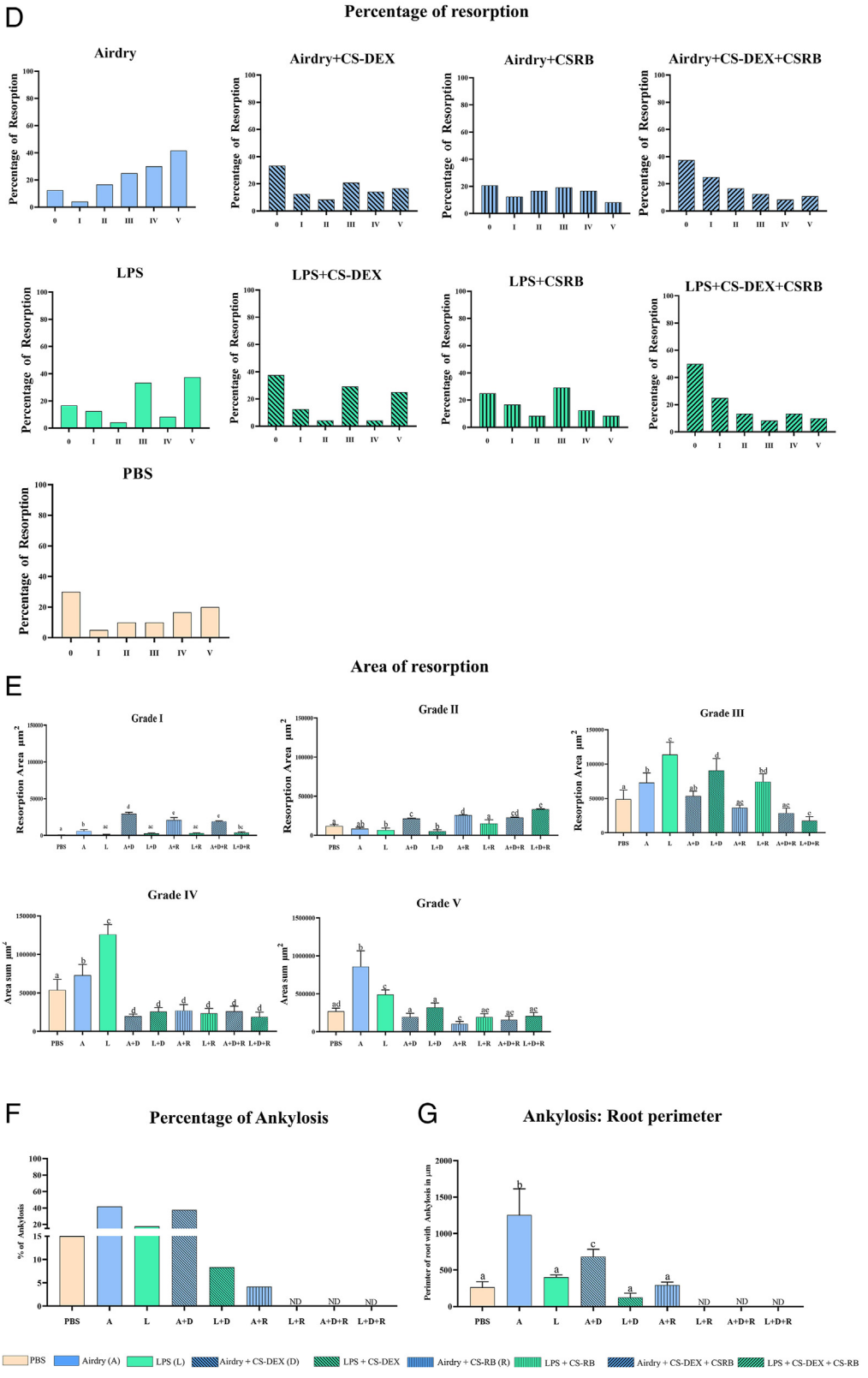

FIGURE 3 – Continued

in used in combination in the animal study. Reduction in the severity and percentage of resorption and ankylosis as measured in histologic analysis further supported the therapeutic potential of CS-DEX/CS-RB in resorption. Periostin, a matricellular protein, plays a crucial role in collagen fibrillogenesis and in maintaining tissue integrity by causing proteolytic activation of lysyl oxidase during

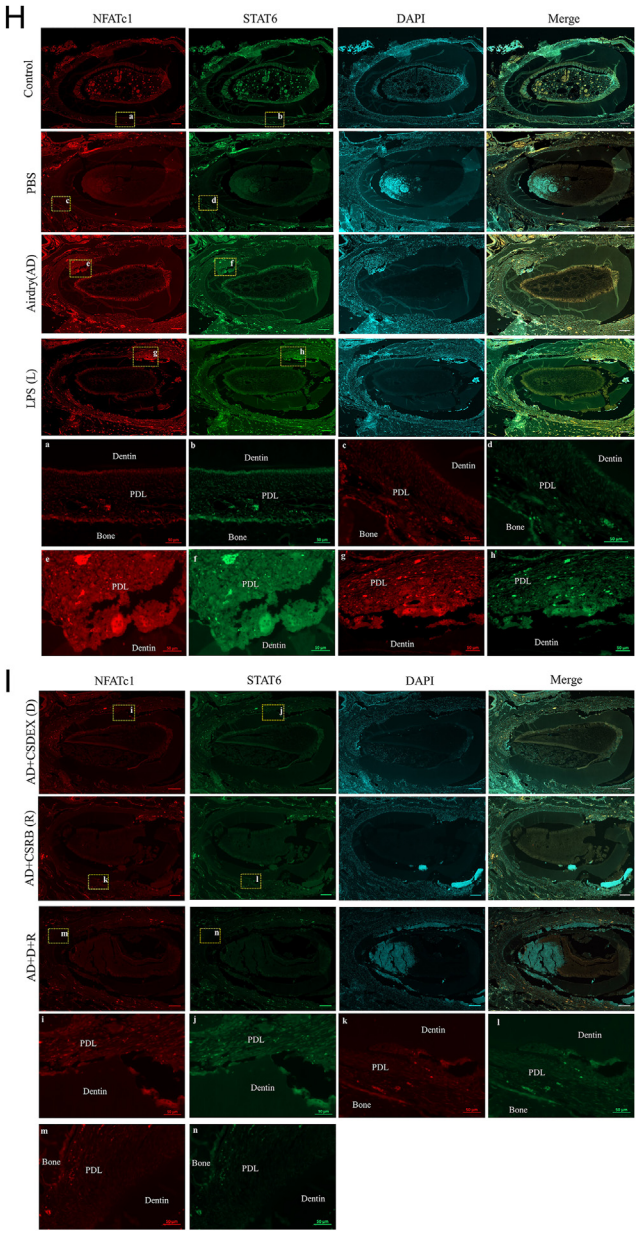

FIGURE 3 – Continued

collagen crosslinking[29]. Periostin thus acts as an adhesive protein that connects these cells and collagen fibers to offer strength and rigidity for biomechanical integrity[29]. PDLF-Mϕ direct coculture treated with CSNP showed significant upregulation of periostin but not with CS-DEX. Similar pattern was observed with TGF-β1 levels. This differential effect of CSNP and CS-DEX could be attributed to the fact that CS-NP was able to significantly upregulate COL1 gene expression compared to CS-DEX. In cellular functions, the expression of cytokines is directly or indirectly interrelated. Mechanical stimuli and TGF-β1 are common stimuli for periostin expression by PDLF[30]. Upregulation of periostin in CSNP coculture groups could be attributed to the increased expression of TGF- β1.

MMP2 and MMP9 are a gelatinase group of proteases of particular interest in remodelling the extracellular matrix and are found in high concentrations in sites of root resorption[31-34]. NPs in *in-vitro* experiments showed upregulation of MMP2 with the downregulation of MMP9. The increased MMP2 levels observed in this study could be attributed to the periostin expression since periostin is shown to induce MMP2 expression via the αvβ3 integrin/ERK signalling pathway[34]. Although MMP2 is commonly known to degrade collagen fiber, it is also reported to play a role in the initiation and progression of collagen fibril growth and in VEGF expression to stimulate angiogenesis[34,35]. MMP2 increase in the current study demonstrates its formative rather than degradative function. MMP9, expressed typically by osteoclasts, is known for eliminating denatured collagen fragments following the action of other MMPs and cysteine proteinases[36]. MMP9 is often found in high levels in teeth with resorbing teeth compared to control[33,36]. In the current study, MMP9 was significantly downregulated in the NP-treated coculture groups compared to untreated ones. MMP2 and MMP9 in CS-DEX/CS-RB groups in the animal studies were comparable levels in healthy control[36]. CSNP treatment further resulted in the upregulation of

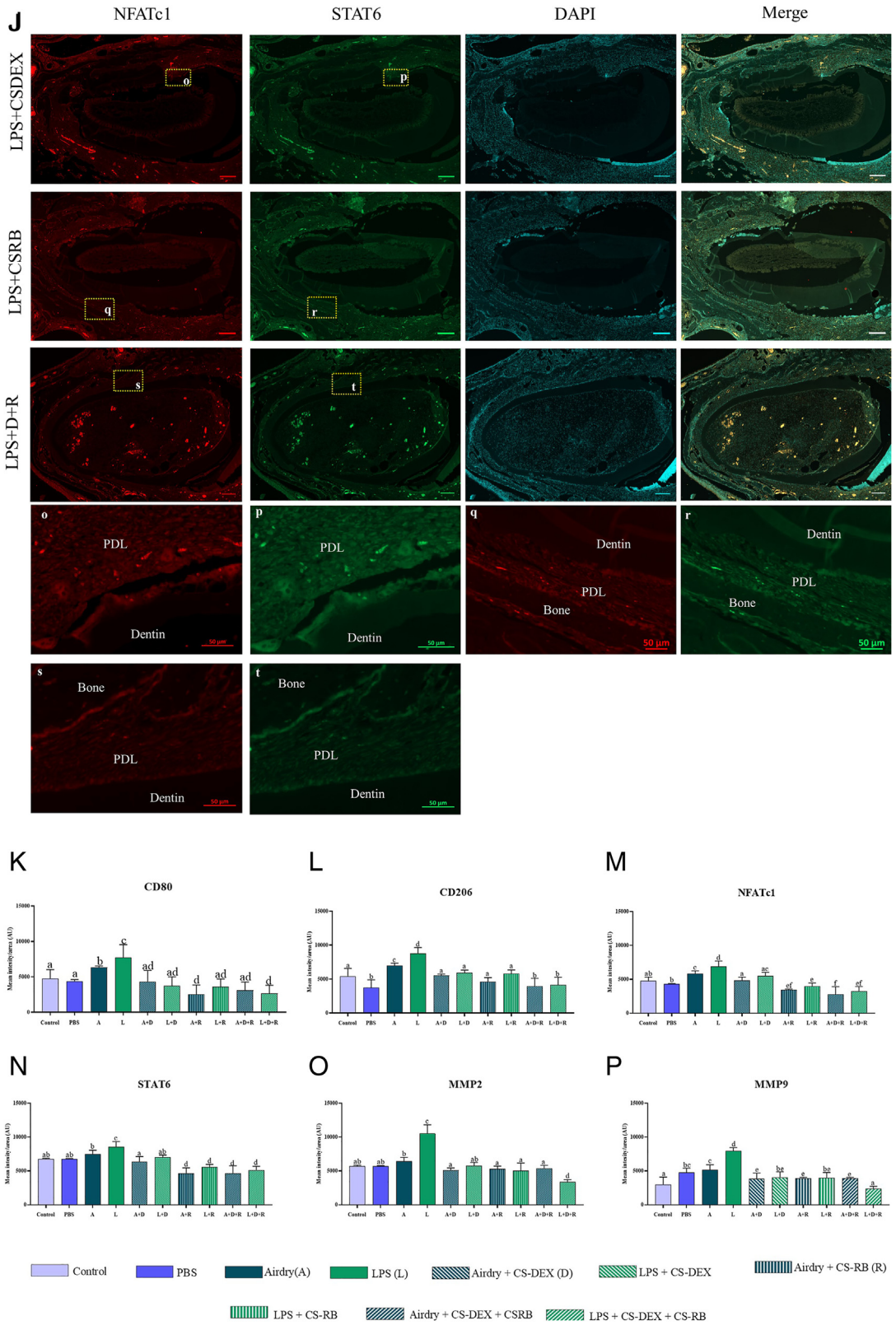

FIGURE 3 – Continued

OPG, a tumor necrosis factor receptor superfamily member and decoy receptor for RANKL in the RANK/RANKL/OPG axis, inhibiting osteoclastogenesis. IL-10 anti-inflammatory cytokines were also significantly upregulated in the CSNP/CS-DEX treatment group. IL-10 and TGF-β1 upregulation observed in the current study is in line with the previous studies of biofilm-treated PDLF-Mϕ

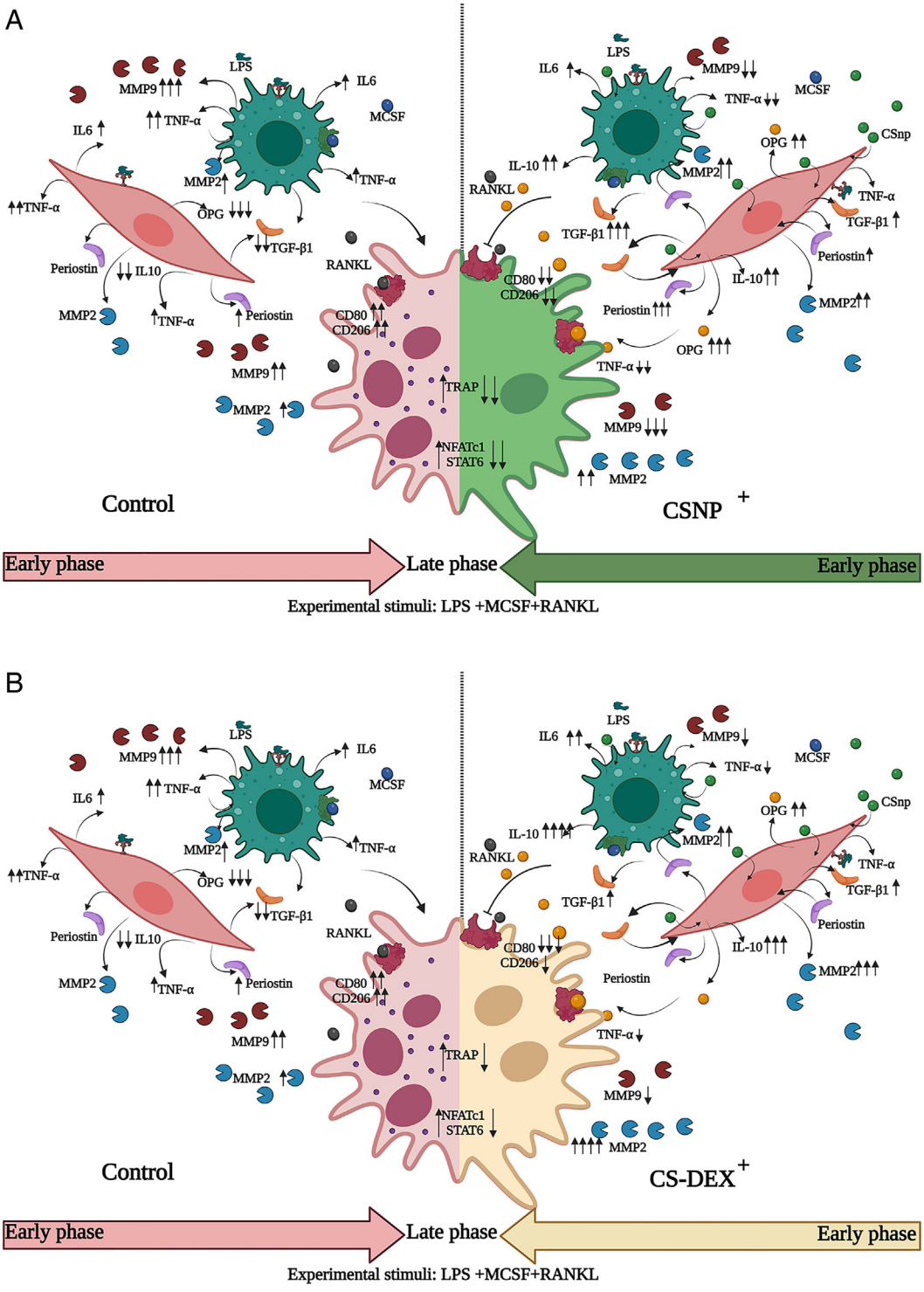

FIGURE 4 – Schematic representation of engineered bioactive nanoparticles guided modulation of PDLF-Mϕ crosstalk in inflammatory resorptive environments. (*A*) and (*B*) represent the cellular interaction in PDLF-Mϕ direct coculture treated with CSNP and CS-DEX in comparison with control (PDLF-Mϕ without nanoparticles). Both CSNP and CS-DEX reduced clastic differentiation of Mϕ in inflammatory resorptive stimuli with temporal and marker specific differences as shown in the schematic above. CS-DEX, dexamethasone conjugated CSNP; CSNP, Chitosan nanoparticles; RANKL, Receptor activator of nuclear factor kappa beta; LPS, lipopolysaccharides; TRAP, Tartrate resistant acid phosphatase; MCSF, macrophage colony stimulating factor; PDLF-Mϕ, periodontal fibroblasts-macrophages.

indirect coculture systems exposed to CSNP[14,15]. TNFα is a potent cytokine secreted by activated macrophages, T cells and fibroblasts[37,38]. It induces osteoclastic differentiation of Mϕ by upregulating c-fms expression and enhancing the RANK's signalling mechanisms. CSNP/CS-DEX successfully reduced TNFα, thereby reducing the proinflammatory microenvironment that favors osteoclastogenesis. While all the cytokines and MMPs showed variation in their expression levels with time, IL-6 levels were

upregulated with NPs. IL-6 is a pleiotropic cytokine with pro- and anti-inflammatory properties and a known potent proinflammatory marker, the increase in CSNP treatment in the current study could be attributed to the feedback mechanism in response to an increase in the anti-inflammatory markers. Based on the current study's findings, the mechanism of engineered bioactive nanoparticles-mediated modulation of PDLF-Mϕ crosstalk in resorptive environments is depicted in Figure 4.

CONCLUSION

The findings from the current study showed that engineered sustained DEX releasing CS-DEX and extracellular matrix modifying CS-RB effectively inhibited osteoclastic differentiation of Mϕ in PDLF-Mϕ direct coculture and reduced resopptive activity and ankylosis *in-vivo* delayed replantation models. This effect could be attributed to the modulation of PDLF-Mϕ crosstalk, demonstrated by the downregulation of CD80, NFATc1, STAT6, and MMP9 expression and upregulation of periostin, IL-10, TGF-β1, and MMP2 activity. Engineering nanosized immunomodulatory bioactive materials chitosan nanoparticles functionalized with photosensitizer and anti-inflammatory agent dexamethasone could serve as potential root surface modification agents to reduce resorptive activity and ankylosis in dental avulsion.

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The authors deny any conflicts of interest related to this study.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found in the online version at www.jendodon.com (10.1016/j.joen.2024.08.006).

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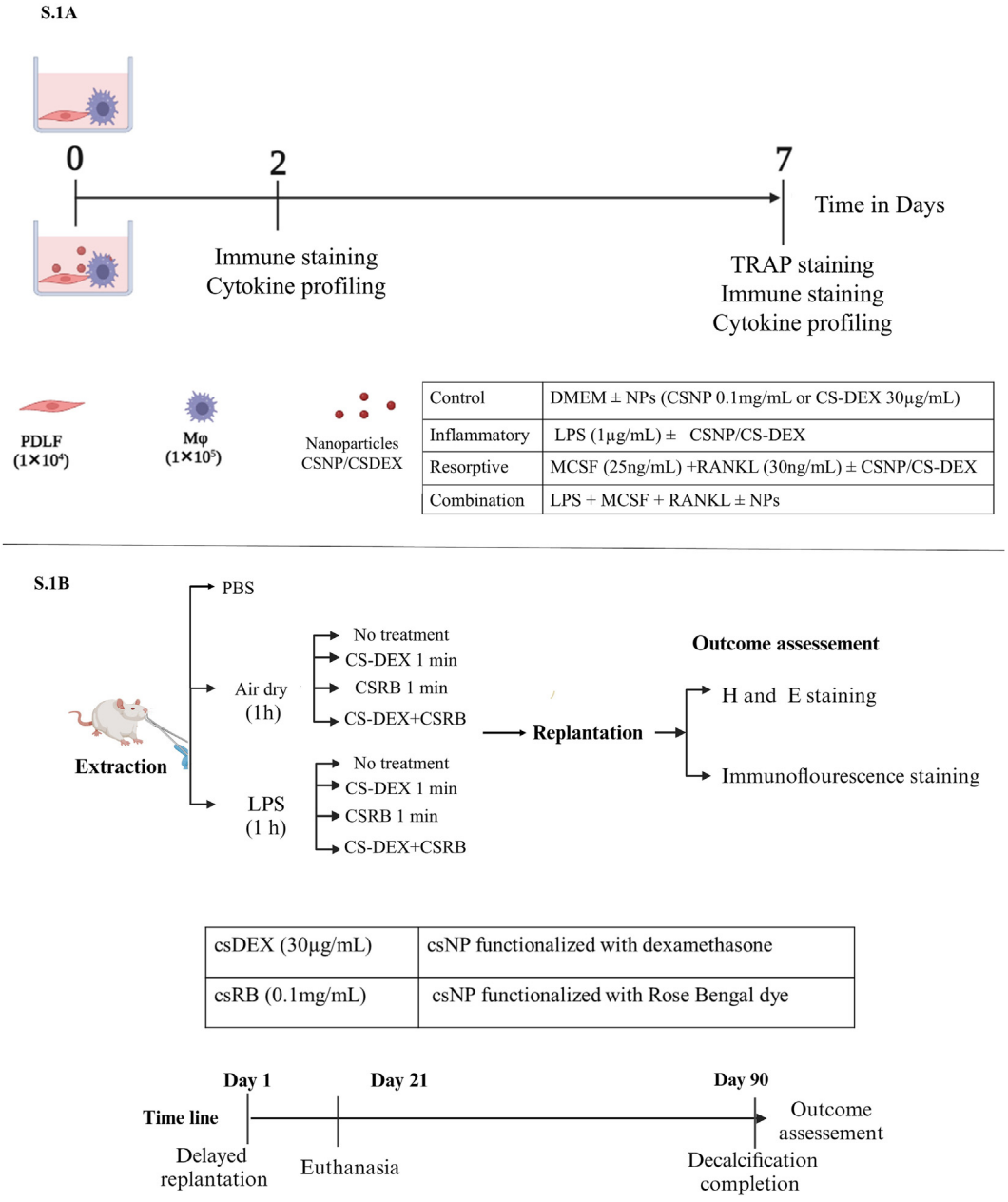
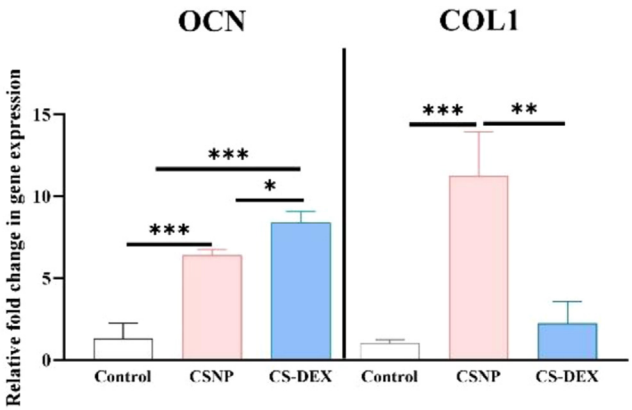


Control	DMEM ± NPs (CSNP 0.1mg/mL or CS-DEX 30μg/mL)
Inflammatory	LPS (1μg/mL) ± CSNP/CS-DEX
Resorptive	MCSF (25ng/mL) +RANKL (30ng/mL) ± CSNP/CS-DEX
Combination	LPS + MCSF + RANKL ± NPs

csDEX (30μg/mL)	csNP functionalized with dexamethasone
csRB (0.1mg/mL)	csNP functionalized with Rose Bengal dye

SUPPLEMENTARY FIGURE S1 – (A): Experimental design of PDLF-Mϕ coculture model exposed to inflammatory, resorptive and combination stimuli with and without CSNP/CS-DEX. (B). Experimental design of delayed replantation model of root resorption with and without nanoparticles (CS-DEX, CSRB) treatment.



SUPPLEMENTARY FIGURE S2 – Relative fold change in gene expression of osteocalcin (OCN) and Type I collagen (COL1) in PDLF monoculture treated with or without CSNP/ CS-DEX for 7 days. Data was analysed with One way ANOVA test with Tukey multiple comparison was performed using GraphPad prism V8.01 and $P < .05$ considered statistically significant. *: $P < .05$, **$P < .01$, ***$P < .001$.

SUPPLEMENTARY TABLE S1 - Stains Used in Immunofluorescence Analysis

Stain	Catalog number	Company	Dilution
DY-594 anti-CD80	305202-BULK CLS100CON	Biolegend	1:150
AF488 anti-CD206	321113	Biolegend	1:150
AF594 anti-NFATc1	sc-7294	Santacruz	1:100
AF488 anti-STAT6	686007	Biolegend	1:200
AF546 anti-MMP9	sc-13520	Santacruz	1:100
AF488 anti-MMP2	sc-13595	Santacruz	1:100
AF647 anti-periostin	Santacruz	sc-398631	1:100

SUPPLEMENTARY TABLE S2 - Primers Used in Gene Expression Assessment

Gene	Forward primer sequence	Reverse primer sequence
GAPDH	5′GGAAGGTGAAGGTCGGAGT-3′	5′TGGAAGATGGTGATGGGATT-3′
OCN	5′TAGTGAAGAGACCCAGGCGCT-3′	5′ATAGGCCTCCTGAAAGCCGA-3′
COL1	5′AGAACTGGTACATCAGCAAG3′	5′GAGTTTACAGGAAGCAGACA3′