CCL2'S IMMUNOMODULATORY ROLE IN THE TREATMENT OF PERIODONTAL DISEASE IN A CANINE MODEL

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Abstract

Periodontitis is an inflammatory disease of the oral cavity induced by bacterial biofilm in a susceptible host. It remains the leading cause of tooth loss in adults worldwide, affecting more than 46% of the adult population in the United States (Eke et al., 2015). Traditionally, this chronic inflammatory disease is treated through non-surgical and surgical therapy, aimed at mechanical debridement of teeth and reduction of bacterial biofilm. Although these methods have proven to be successful in reducing inflammation and disease progression, they are costly, time consuming and painful for patients to tolerate. In recent years, the focus has shifted towards therapies involving host modulation as an adjunct to non-surgical and surgical treatment. The purpose of this investigation was to evaluate the effects of the C-C motif chemokine ligand 2 (CCL2) on host immunomodulation in a canine ligature induced periodontitis model.

Six female beagle dogs were used for the study. The subjects were randomly assigned to one of four treatment groups: healthy control, ligature induced periodontitis—untreated, ligature induced periodontitis treated with blank microparticles (MPs), and ligature induced periodontitis treated with recombinant human (rh) CCL2 MPs. The ligatures were secured to the subjects' mandibular left and right first molar (M1) and fourth premolar (P4). Controlled release blank

MPs or rhCCL2 containing MPs were injected into the marginal gingiva of the subjects at baseline (week 0), week 4, and week 8. Clinical parameters of probing depths and bleeding on probing (BOP) were assessed at baseline, 4 weeks, 8 weeks and 12 weeks. The dogs were sacrificed at week 12 and mandibular biopsies were harvested for micro-computed topography (CT) analysis.

The administration of rhCCL2 MPs, even in the presence of chronic inflammation, provided protection against attachment loss as measured by changes in probing depth and alveolar bone loss compared to the untreated groups. We can conclude from this study that rhCCL2 MPs successfully modulated the immune response to periodontal pathogens in the ligature induced periodontitis canine model, resulting in attachment levels comparable to the healthy control group.

Table of Contents

1.0 Introduction

1.1 The Periodontium

The periodontium consists of four components, the alveolar bone, periodontal ligament, cementum, and gingival tissues. These anatomical structures provide support for our teeth and play a critical role in the maintenance of periodontal health and a barrier against periodontal disease. When periodontal pathogens invade the periodontium, the integrity of these components is challenged, which over time can result in irreversible damage and ultimately tooth loss.

The gingival tissues are the most superficial component of the periodontium and form the initial barrier against microbial infiltration. The gingiva is composed of the epithelium layer, which is at the surface, and the connective tissue layer beneath it. The epithelium is further divided into the three categories, the oral, sulcular and junctional epithelium (JE). The junctional epithelium (JE) acts as the barrier between the gingival sulcus and the deeper layers of the periodontium, therefore, playing a crucial role in the maintenance of periodontal homeostasis. More specifically, the JE forms a tight cuff like connection to the cementum via hemidesmosomes in its basal laminal layer (J. Lindhe & Lang, 2015). In healthy conditions, this attachment behaves as a barrier against pathogenic bacteria from affecting the deeper layers of the periodontium, such as the alveolar bone, periodontal ligament and cementum. The JE has several unique characteristics that allow it to function as the gatekeeper of periodontal homeostasis. Firstly, the

JE cells undergo rapid cell turnover allowing them to be quickly repaired or replaced in the presence of a microbial challenge. Secondly, the JE cells have wide intercellular spaces which allow for migration of immune defense cells such as polymorphonuclear (PMN) phagocytes and macrophages (monocytes) (Pöllänen, Salonen, & Uitto, 2003). In addition to recruiting and mobilizing these immune cells, the JE cells have shown their own endocytic activity making them critical to the overall innate immune response (Cho & Garant, 2000). In all, the JE not only acts as a physical barrier protecting tooth supporting tissue, but these cells actively facilitate the recognition and subsequent elimination of periodontal pathogens.

The periodontal ligament (PDL) is a thin layer of tissue which supports the tooth in its socket and behaves mainly as the "shock absorber" transmitting occlusal load evenly to the bone and providing proprioception to the brain. These specialized connective tissue cells also direct remodeling of bone and cementum in response to external loads (Nanci & Bosshardt, 2006). The PDL has both cellular and non-cellular components. The cellular elements include fibroblasts, osteoblasts, and cementoblasts responsible for connective tissue and bone homeostasis. The immune cells, such as neutrophils, macrophages, lymphocytes and mast cells, are responsible for fighting bacterial challenge. The epithelial cells, also called "epithelial rests of malassez," are remnants of cells responsible for tooth root formation (Nanci & Bosshardt, 2006). The non-cellular components refer to the principle fibers of the PDL, which are mainly collagen type I and III (Huang, Ohsaki, & Kurisu, 1991). These principle fibers form calcified terminal ends known as Sharpey's fibers as they insert into the cementum on one side of the PDL and the alveolar bone on the other side.

The cementum is an avascular hard tissue that forms the outer most layer of the tooth root and presents itself in two forms, acellular and cellular. The acellular cementum exists in the

cervical portion of the tooth root which forms an intimate connection to the PDL via the Sharpey's fibers. The cellular cementum constitutes the apical portion of the tooth root characterized by cementocytes (cells trapped in lacunae). Cementocytes are responsible for minor repair of any resorption of collagen fibers by continued deposition to keep the attachment apparatus intact (Diekwisch, 2001).

The alveolar bone encases the tooth sockets and contains the lamina dura, a lining of compact bone around the roots of the teeth allowing attachment to the cementum via the PDL. This alveolar bone is known as "bundle bone" since it is the site of PDL fiber insertion and home of intrinsic collagen fiber bundles running parallel to the socket walls. The alveolar bone is constantly undergoing remodeling in response to physiological masticatory forces applied to the teeth (Verna, Zaffe, & Siciliani, 1999).

1.2 The Pathogenesis of Periodontal Disease

Under healthy conditions, the periodontium can maintain an environment free from inflammation and support the teeth during function. However, in the continual presence of periodontal pathogens there is a prolonged inflammatory response that can compromise all four components of the periodontium, resulting in periodontal disease and subsequent attachment loss. For decades, we have known that dental plaque biofilm (bacterial biofilm) is the major etiologic factor in the initiation of periodontal disease, marked by the breakdown of the periodontium (J. Lindhe, Hamp, & Loe, 1975; Lovegrove, 2004). Initially, the bacterial biofilm is mainly composed of commensal (non-disease causing) microbiota, which can accumulate in as little as 48 hours if oral hygiene is stopped (J. Lindhe et al., 1975). As the plaque builds up at the gingival margin, the

increase in commensal microbes begins to alter the local homeostasis and initiates vasodilation and inflammation of the gingival tissues. In its early stage, this inflammatory response is limited to the gingiva and is reversible, referred to as gingivitis (Murakami, Mealey, Mariotti, & Chapple, 2018). If this bacterial biofilm continues to form on the tooth surface and not adequately removed, it can transform into more aggressive periodontal pathogens such as *aggregatibacter actinomycetemcomitans* (Aa), *porphyromonas gingivalis* (Pg), *tannerella forsythia* (Tf), *treponema denticola* (Td), *fusobacterium nucleatum* (Fn) and many more pathogens of the facultative and obligate anaerobic family (Haffajee et al., 1997). For instance, Pg has been shown to directly disrupt the integrity of the JE and negatively impact its ability to act as a barrier (Katz et al., 2002). The propagation of these periodontal pathogens past the JE and into the underlying connective tissue results in the chemotaxis and expansion of immune cells such as PMNs and macrophages, which in turn secrete pro-inflammatory cytokines (IL-6, TNF-a, IL-1 B), chemokines and proteases (MMPs) which then break down the connective tissue to compartmentalize the pathogens. If these pro-inflammatory cytokines persist with no resolution, they will ultimately impact the alveolar bone homeostasis favoring osteoclastic activity (bone loss) over osteoblastic activity (bone formation). This uncoupling of bone formation from bone resorption results in net bone loss, which marks the transition from gingivitis to periodontitis (Graves, Li, & Cochran, 2011).

Although periodontal pathogens initiate the inflammatory cascade that ultimately leads to alveolar bone loss, these pathogens do not directly resorb the periodontium. Alveolar bone loss is driven by increased osteoclastic activity based on an imbalance between the receptor activator of nuclear factor kappa-B ligand (RANKL) and its decoy receptor osteoprotegrin (OPG) (Boyce & Xing, 2008). When pro-inflammatory cytokines are secreted in the inflamed periodontal

connective tissue, it results in the stimulation of osteoblasts, bone marrow stromal cells, and PDL fibroblasts to secrete RANKL, which binds to its receptor RANK on osteoclasts precursors. OPG would normally compete with RANK for the RANKL protein, however under pro-inflammatory environments RANKL production is much higher than OPG. As a result, high quantities of RANKL bind to RANK and induce the differentiation and maturation of osteoclasts resulting in inflammation induced alveolar bone loss (Graves et al., 2011). In addition to the propagation of alveolar bone loss, activated immune cells also release enzymes such as matrix metalloproteinases (MMPs) which further destroy PDL collagen fibers allowing the inflammation to spread to deeper layers of the periodontium resulting in further attachment loss (Kurgan & Kantarci, 2018). Collectively, the progression from periodontal health to periodontal disease is periodontal biofilm induced inflammation resulting in destruction of the soft and hard tissue components of the periodontium.

1.3 The Host Response

It is well established that the uncontrolled host response in the presence of bacterial biofilm is the major mechanism underlying the tissue breakdown and attachment loss associated with periodontal disease. The host response is essential in the transformation of a gingivitis lesion, which is reversible, to a periodontal disease lesion, which is irreversible. This progression of disease is mediated by a wide array of inflammatory cytokines such as IL-6, TNF-α, IL-17A and chemokines, such as CXCL12 (Graves et al., 2011), which are responsible for ultimately transforming the immune response from the innate immune system to the acquired immune system.

Conceptually, these cytokines and chemokines function to limit periodontal infection by activation and recruitment of immune cells such as phagocytes to defend against the bacterial insult (Parameswaran & Patial, 2010; Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011). However, these immune cells can induce tissue destruction by stimulating production of proteolytic enzymes and inducing osteoclastic differentiation, thus promoting periodontal tissue damage, disease progression and dysbiosis (Cochran, 2008). In fact, the overactive host immune response accounts for much more soft and hard tissue breakdown when compared to the tissue destruction caused directly by the periodontal pathogens via release of proteolytic enzymes and cytotoxic byproducts (Cekici, Kantarci, Hasturk, & Van Dyke, 2014).

Several different immune cells play an important role in the destructive periodontal inflammatory response, in particular, macrophages and T-cells are crucial in the pathogenesis of periodontal disease. When periodontal soft tissue biopsies were obtained from chronic periodontitis patients, macrophages comprised 6% of all the immune cells that were present (Carcuac & Berglundh, 2014). When compared to healthy tissues, the percentage and total value of macrophages was also higher in both human and murine disease models (Gemmell, McHugh, Grieco, & Seymour, 2001; Yu et al., 2016). Human chronic periodontitis lesions also showed significantly higher levels of T cells and the pro-inflammatory cytokine they produce (IL-6, TNF-α, IL-17A) (Lester, Bain, Johnson, & Serio, 2007; Ohyama et al., 2009). Furthermore, the severity of human periodontitis seems to be directly correlated with the quantity of these proinflammatory cytokines.

Within the periodontium, macrophages are active participants of the inflammatory response, both in its initiation and in its propagation. These cells exhibit a wide spectrum of phenotypic subsets with M1 (classically activated) and M2 (alternatively activated) phenotypes

representing two ends of the spectrum (Mantovani et al., 2004). The stage and severity of the inflammatory process often determines which phenotype is favored. For example, the M1 macrophage is responsible for orchestrating the initial pro-inflammatory response for host defense in the presence of bacterial pathogens. The M2 macrophage phenotype promotes an anti-inflammatory response leading to resolution of disease and tissue repair following the eradication of the bacterial insult (Mantovani et al., 2004). Because of this diversity in phenotype and function, macrophages are important to not only the initiation and progression of inflammation, but also its resolution (Oishi & Manabe, 2018). Thus, modulating the phenotype of macrophages can play an important role in the successful treatment of periodontal disease.

Over the last few years, several studies have investigated the macrophage phenotype response as a therapeutic target to treat periodontal disease. A 2018 study was able to use Rosiglitazone (PPAR γ agonist) to induce M2 macrophage phenotype in the murine model. This resulted in inhibition of bone loss and stimulation of bone formation in the murine periodontium (Viniegra et al., 2018). Another study completed in 2020 was able to show that dental pulp stem cell exosomes injected locally can facilitate periodontal bone healing by promoting the M2 macrophage polarization (Shen & Gaffen, 2008). Based on our current understanding of the macrophage types and their roles in the different stages of inflammation, it has become apparent that the modulation of macrophage phenotypes can be a promising target for future pharmaceutical intervention when treating periodontal disease.

1.4 Traditional Periodontal Therapy

Our current approach to treating periodontal disease is aimed at reducing the bacterial biofilm in the periodontium through mechanical debridement of the subgingival root surface (scaling and root planing). This is sometimes in conjunction with antiseptics and antibiotics (Sanz et al., 2015; Teles & Teles, 2009). In patients who do not show resolution of inflammation, surgical intervention is often needed to gain access to the more apical regions of the root surface where the bacterial biofilm persists—despite completed initial therapy. Periodontal surgery, also known as the "phase II treatment", can include resective osseous surgery and guided tissue regeneration. Osseous surgery aims to eliminate defects in the alveolar bone and guided tissue regeneration aims to gain clinical attachment by excluding the epithelium during the healing phase. These therapies have a proven track record in reducing periodontal disease, however, they can be time consuming, costly and painful for patients to tolerate (Heitz-Mayfield, Trombelli, Heitz, Needleman, & Moles, 2002; Needleman, Giedrys-Leeper, Tucker, & Worthington, 2001).

Adjunctive systemic antibiotics have been shown to be improve the outcome of periodontal therapy in certain situations; in particular, very severe cases of periodontal disease that present with advanced bone loss (Haffajee, Socransky, & Gunsolley, 2003). Long term antibiotic use is, however, contraindicated due to growing concern over antibiotic resistance (Ventola, 2015; Walters & Lai, 2015). Some success has been achieved with local antimicrobial agents, such as Arestin (minocycline) and PerioChip (chlorhexidine), which are administered directly into the gingival sulcus in the presence of inflammation (Paquette, Ryan, & Wilder, 2008). Even with thorough root debridement and meticulous surgical intervention, many patients present with recurrent or refractory periodontal disease. This leads to clinicians seeking alternative adjuncts to our traditional methods of periodontal therapy.

1.5 Periodontal Host Modulatory Therapies

Host modulation therapy (HMT) refers to treatments targeting immune cell pathways with the desired outcome of altering the host's response to inflammation. Although this therapeutic modality is relatively new to the field of dentistry, it has been widely used for other chronic inflammatory diseases such as rheumatoid arthritis (Guo et al., 2018).

Over the last few decades, our understanding of the pathogenesis of periodontal disease has evolved from considering it as merely a microbial plaque induced disease to a multifactorial condition in which the host immune response to the periodontal pathogens is instrumental in disease initiation and progression (Bartold & Van Dyke, 2013) . With this new understanding, there has been an incentive to explore various host modulation therapies (HMT) aimed at improving the outcomes of current traditional periodontal therapies. Ultimately, HMTs are designed to promote active healing process while mitigating the progression of inflammation in the periodontium. One such example of HMT, is sub-antimicrobial dose Doxycycline, sold as the medication "Periostat," and has been FDA approved for treatment of periodontal disease. Periostat contains 20mg of doxycycline given twice daily for a period of time ranging from 3-9 months. Its usage is aimed at reducing clinical attachment loss. Doxycycline is a well-known broad spectrum bacteriostatic antibiotic, however, when given at these sub-antimicrobial doses it provides an inhibitory effect on matrix metalloproteinases (MMPs) that are responsible for tissue breakdown and propagation of inflammation in the presences of periodontal pathogens (Peterson, 2004). When Periostat was administered in conjunction with mechanical plaque debridement, patients with periodontal disease showed statistically significant improvements in clinical parameters (Caton, 1999). Systemic host modulatory therapies have shown some clinical benefits, however

their overall use may be limited due to undesirable systemic side effects, compliance, and the duration you can stay on the medications.

Due to the limitations of systemic HMT, researchers have developed new local host modulatory therapies to diminish specific immunological pathways that can contribute to periodontal pathogenesis, while also upregulating the pathways that promote inflammation resolution. One such target of local HMT are omega-3-polyunsaturated fatty acids (PUFAs), known as resolvins, which are a class of lipid mediators responsible for resolution of the intrinsic inflammatory pathway (Serhan, Chiang, & Van Dyke, 2008). Resolvins function by suppressing pro-inflammatory cytokines, inhibiting neutrophil recruitment, and promoting macrophage phagocytosis of apoptotic neutrophils. For example, RevE1 is a member of the resolvin family that is able to mitigate RANKL induced maturation of pre-osteoclastic cells and also increase the OPG production by osteoblasts favoring an environment for bone repair instead of bone destruction (Gao et al., 2013; Herrera et al., 2008). Local treatment with RevE1 has shown to reduce periodontal break down, promote repair and even reverse the microbial dysbiosis in animal models (H. Hasturk et al., 2006; Lee et al., 2016).

Local delivery of immune-modulatory peptides has emerged as another promising approach for the resolution of inflammation in periodontally infected tissues. These peptides (e.g. cytokines and chemokines) can be packaged into time released carriers and delivered directly into the diseased periodontium to alter the local environment favoring the resolution of inflammation. Proof of concept animal studies in both mice and canines have shown the efficacy of these peptides, in particular the C-C motif chemokine ligand family (CCL). For example, localized release of CCL22 into the periodontal tissue of mouse and canine subjects was able to inhibit alveolar bone loss in the presence of periodontal disease (Glowacki et al., 2013). CCL22 functions

to recruit T-regulatory cells (Tregs) which in turn are capable of producing anti-inflammatory and reparative cytokines such as IL-4, IL-10, and TGF-β (Kim et al., 2007). More recently, C-C motif chemokine ligand 2 (CCL2) was able to protect against alveolar bone loss in both *porphyromonas gingivalis* (Pg) and ligature induced periodontitis in the murine model (Zhuang et al., 2019). CCL2 functions by shifting the ratio of macrophages from the M1 inflammatory phenotype to the M2 anti-inflammatory phenotype. CCL2 has been shown to not only function as a potent chemoattractant for the monocyte/macrophage lineage, but also as an M2 phenotype polarization inducer. This shift in macrophage phenotype would alter the cytokine profile by reducing the production of pro-inflammatory cytokines and increasing the production of anti-inflammatory cytokines, resulting in reduced osteoclastic differentiation and less alveolar bone destruction (Zhuang et al., 2019).

Conceptually, CCL2 therapy would reduce the inflammatory response in the periodontal tissue even in the presence of bacteria such as in ligature induced periodontitis. If this hypothesis is true, it would mean that local administration of CCL2 in active periodontal disease sites would result in less alveolar bone destruction and less clinical attachment loss compared to untreated sites.

1.6 Specific Aims

The purpose of this investigation was to evaluate the effects of local delivery of CCL2 on the canine host inflammatory response in the presence of ligature induced periodontitis. Clinical attachment loss and extent of inflammation was measured by probing depths and bleeding on probing in the treatment group compared to healthy control, blank, and untreated groups.

Additionally, mandibular biopsies were collected at the conclusion of the study for micro-CT analysis to quantify the extent of alveolar bone loss.

2.0 Materials and Methods

2.1 Fabrication of Sustained Release Microparticles

Sustained release CCL2 microparticles (MPs) were fabricated by encapsulating recombinant human CCL2 (rhCCL2) protein (R&D systems) into Poly-Lactic-co Gylcolic Acid (PLGA) (Sigma) using a standard water-oil-water double emulsion technique as described previously (Glowacki et al., 2013). The first aqueous phase consisted of 200 µl of 25 µg of rhCCL2 with 1% bovine serum albumin (BSA) and 5 mM NaCl. The oil phase was made by dissolving 200 mg PLGA polymer (lactic:glycolide 50:50, Mwt 7000 – 17000) in 4 mL dichloromethane (DCM). The first aqueous and oil phases were sonicated together to obtain an emulsion that was then homogenized with 60 ml of 2% polyvinyl alcohol with 50 mM NaCl (second aqueous phase) to make the second emulsion. The homogenate was then added to 80 ml of 1% polyvinyl alcohol with 50 mM NaCl and stirred at 600 rpm for 3 hours to allow for evaporation of DCM. Next, the MPs were collected and washed, then resuspended in 5 ml of deionized water and flash frozen in liquid Nitrogen before lyophilization. Blank (unloaded) PLGA MP were also fabricated, without recombinant protein, following the same protocol.

2.2 Canine Periodontal Disease Model

Ligature induced periodontitis was caried out in a canine model using six, female, beagle dogs; approximately 12 months of age. Each subject had four treatment teeth: the mandibular left

and right fourth premolar (P4) and first molar (M1). The subjects were randomly assigned to one of four treatment groups: (see Table 1) healthy control with no ligatures, ligature induced periodontal disease—untreated, ligature induced periodontal disease treated with blank MPs, and ligature induced periodontal disease treated with rhCCL2 MPs.

All procedures on the canines were completed under general anesthesia (0.1-025mg/kg Acepromazine and 1-3% Isoflurane) with mask and oral intubation. At the beginning of the study, all subjects' teeth in the treatment quadrants were scaled to establish a level of "health". Oral hygiene measures of twice daily tooth brushing were instituted following scaling and continued for the remainder of the study. Two weeks following initial scaling, the canines in Groups #2-4 had 4-0 silk ligatures tied to their mandibular M1 and P4 teeth, bilaterally. This time point was recorded as baseline (week 0). Ligatures were secured by creating a small groove in the height of contour of the teeth, with a carbide round bur, and tied in place. Additionally, the dogs with ligatures were injected with either blank microparticles, particles containing rhCCL2, or left untreated, Particle injections were completed by injection of 50uL of MPs into the gingiva surrounding the treatment teeth. The MPs were injected at six sites on the teeth, three on the facial gingiva (mesiobuccal, straight buccal, distobuccal) and the same three sites on the lingual gingiva (mesiolingual, straight lingual, and distolingual respectively). MPs were injected using insulin syringes. The particle injections took place at baseline, 4 weeks, and 8 weeks. A schematic of the study design can be seen in Figure 1.

Throughout the duration of the study, ligature placement was verified every two days to confirm no ligatures were lost prematurely. If a subject was missing its ligature, it was replaced

within two days. The canines were placed on a soft diet throughout the study to promote plaque accumulation and prevent ligature dislodgment. No adverse reactions were encountered in any of the subjects during the duration of the study. At weeks 0, 4, 8, and 12 clinical measurements of bleeding on probing (BOP) and probing depths (PDs) were recorded and clinical photographs were taken. At the conclusion of the study (12 weeks) the subjects were sacrificed and mandibular biopsies were taken from each subject for use in micro-CT analysis.

2.3 Micro-computed tomography

At the end of conclusion of the study (week 12), canine mandibles were harvested and fixed overnight in 10% neutral buffered formalin then transferred to 70% ethanol for scanning with a micro-computed tomography system (Scanco μCT 50, Scanco Medical). A resolution of 10μm voxel size, 55KVp, 0.36 degrees rotation step (180 degrees angular range) and a 1200 ms exposure per view were used. All scans were reoriented with DataViewer software (GE Healthcare) to a standardized orientation guided by pre-defined anatomical landmarks. For bone loss evaluation, reoriented images were used to measure the distance between the cementoenamel junction (CEJ) and the level of the alveolar bone crest (ABC) of the mandibular first molar and mandibular fourth premolar on the mesial, distal, buccal and lingual aspects using CTAN software (Bruker). For mesial and distal measurements, the CEJ to ABC distance were measured on 8 measurement slices with a distance interval of 40 μm in between. For the buccal and lingual measurements, the CEJ to ABC distance were measured on 20 measurement slices with a distance interval of 30 μm in between. The average measurements on each aspect on the ligated side were normalized to the

corresponding averages on the healthy side of the same maxilla. The investigator/personnel conducting the measurements was blinded to the treatment received.

2.4 Sample size, power calculation, and statistical analysis

The number of animals used in this study was calculated from a power analysis performed on previously reported results and preliminary data (Glowacki et al., 2013; Zhuang et al., 2019). Specifically, for disease amelioration (or efficacy of treatments), using alveolar bone loss as the primary outcome variable a power value of 0.8 was recommended. For fixed-effects one-way ANOVA, with α=0.05 and β=0.2, we would need a total of 6 dogs to achieve statistical significance.

Cementoenamel junction (CEJ) to alveolar bone crest (ABC) measurements were conducted by randomized and blinded sample analysis. Furthermore, statistical analyses comparing multiple groups: age match control animals, ligated but untreated dogs, blank PLGA MP treated dogs and the experimental group (rhCCL2 MP treated dogs) was performed by first ensuring normal alveolar bone loss distributions using the Shapiro-Wilk test. Statistical analysis was then performed using one-way ANOVA followed by Tukey HSD multiple comparisons test for significance. Differences were considered statistically significant if $p<0.05$ and all statistics were performed by utilizing GraphPad Prism software.

3.0 Results

3.1 Characterization and Release Profile of CCL2 MPs

The characterization and release profile of sustained delivery recombinant mouse CCL2 (rmCCL2) MP was determined by a previous study (Shehabeldin, 2021). In their study it was demonstrated with SEM scanning of the CCL2 MP that the uniformly spherical MP contained varying degrees of surface porosity (Figure 2A) (Shehabeldin, 2021). The volume impedance measurement showed that the average particle size was 21.49-±10.02 μm (Shehabeldin, 2021) (Figure 2B). The aforementioned study also analyzed the release profile of the PLGA MPs containing recombinant mouse CCL2. The same carrier, PLGA MPs, were utilized in this study, however, the MPs contained rhCCL2 instead of rmCCL2. The sustained delivery release profile for rhCCL2 can be seen in Figure 3. This illustrates the 36 days cumulative ELISA release profile. It was demonstrated that release of rhCCL2 protein from the PLGA MP started with a burst release from days 0-5, followed by a gradual steady release for the remainder of the timeframe.

3.2 Effects of CCL2 on Bleeding on Probing

Bleeding on probing (BOP) was measured at six points on each tooth; straight buccal (SB), mesiobuccal (MB), distobuccal (DB), mesiolingual (ML) distolingual (DL), and straight lingual (SL). Sites were awarded a value of a “0” or a “1”; “0” meaning no sign of bleeding at gentle probing and a “1’ indicated positive for blood in the sulcus following periodontal probe insertion.

These values were recorded at baseline (week 0), week 4, week 8, and week 12. The particle injections were completed at week 0, week 4, and week 8. For each time point, BOP values were averaged as a percentage per treatment group (see Figure 5).

Clinical photographs capturing the degree of BOP amongst the four groups, taken at week 12, can be seen in Figure 4. A noticeable difference can be discerned in the degree of inflammation in the ligated groups when compared to the healthy control; with the untreated group demonstrating the most significant amount of clinically evident BOP.

Comparison amongst the groups demonstrated that at baseline, there was no statistically significant difference amongst the groups. At week 4 and week 8, there was a statistically significant difference between the healthy control group and all three ligature groups. At week 4, healthy control had an average bleeding on probing measurement of 12.5% compared to 87.5% in the untreated, 91.7% in the blank MPs and 77.08% in the rhCCL2 group. At week 8, healthy control had an average bleeding on probing measurement of 37.5% compared to 87.5% in the untreated, 79.2% in the blank MPs and 93.8% in the rhCCL2 group.

At the conclusion of the study, week 12, a statistically significant difference was only noted between rhCCL2 and healthy control (66.7% vs 33.3% respectively). However, there was no difference observed between rhCCL2, blank MPs and untreated (66.7% vs 50% vs 62.5% respectively), suggesting that all groups with ligatures demonstrated BOP, regardless of intervention (Figure 5).

3.3 Effects of CCL2 on Change in Probing Depths

Gingival probing depths were recorded by measuring the distance from free gingival margin to the base of the sulcus. Probing depths were measured at six points on each tooth; straight buccal (SB), mesiobuccal (MB), distobuccal (DB), mesiolingual (ML) distolingual (DL), and straight lingual (SL). These values were gathered at baseline (week 0), week 4, week 8, and week 12.

Change in probing depth was calculated by subtracting the probing depth at weeks 4, 8, and 12 from baseline (week 0). These values were calculated as a change in probing depth per subject and then averaged to achieve a change in probing depth per treatment group (Figure 6).

As expected, all four groups showed in increase in probing depths from baseline with the onset of periodontal disease. However, CCL2 administration demonstrated statistically significant change in probing depths compared healthy control at week 12 (0.53mm vs 0.50mm), suggesting that the CCL2 microparticles protect against attachment loss even in the presence of ligature induced periodontal disease. Interestingly, at the conclusion of the study (week 12), CCL2 probing depths were significantly ($P<.01$) less than the untreated and blank MPs (1.05mm & 1.23mm respectively), even outperforming the healthy control by a small margin when all groups are compared.

3.4 Micro-CT Analysis

To quantify alveolar bone loss, micro-CT analysis was performed on the canine mandibles harvested when the dogs were sacrificed at the conclusion of the study (week 12). The images were reoriented using anatomical landmarks and vertical bone loss was measured as the distance between the cemento-enamel junction (CEJ) and the alveolar bone crest (ABC) (**Error! Reference source not found.**). Alveolar bone loss in the CCL2 and untreated groups were compared to the healthy control. At week 12, the micro-CT analysis revealed 0.74mm of bone loss (from the CEJ-ABC) in the untreated group, compared to 0.52mm in the CCL2 group and 0.35mm in the healthy group. Statistical analysis (one-way ANOVA) confirmed that untreated group had significantly more alveolar bone loss compared to the healthy group ($P<.01$) as expected. Comparing alveolar bone loss in the untreated group to the CCL2 group, revealed a P Value of 0.0544. Although not statistically significant ($P<.05$), it does suggest that CCL2 MPs can provide some protection against periodontally induced alveolar bone loss. Interestingly, when comparing the alveolar bone loss values in the healthy control to the CCL2 group (0.35mm vs 0.52mm), there is no statistical difference with a P value of 0.0739. This implies that the use of rhCCL2 MP is statistically much closer to the healthy control than the untreated control when it comes to alveolar bone loss from ligature induced periodontitis.

4.0 Discussion

The role of the immune system in the pathogenesis of the periodontal disease has been well documented in the last quarter century. During that time the scientific community has more accurately discerned the role of specific immune cell types and how they propagate the inflammatory response to periodontal pathogens, ultimately resulting in clinical attachment loss. Macrophages are responsible for a wide variety of functions during both health and disease states, however during periodontal disease they appear in greater numbers and are key players in the transition from the innate immune response to the adaptive immune response (Gemmell et al., 2001; Yu et al., 2016).

The bone marrow contains hematopoietic stem cells that give rise to monocyte-derived macrophages which will ultimately infiltrate tissues to protect against foreign pathogens. Once in the tissues, macrophages can behave either as pro-inflammatory M1 phenotypes or as anti-inflammatory M2 phenotypes based on how they are activated by their local environments (Martinez & Gordon, 2014). The classically activated M1 phenotype eliminate pathogens by phagocytosis, while also activating polymorphonuclear neutrophils (PMNs) and T cells to produce pro-inflammatory cytokines; such as IL-1β, IL-6 and TNF-α (Sima & Glogauer, 2013). M1 macrophages also promote ostoeclasitc activity by activating the Th1 response and stimulating pre-osteoclastic cells. This results in elevated levels of RANKL which promotes further osteoclastic activity and eventual alveolar bone loss (Adamopoulos & Mellins, 2015). The M2 macrophage phenotype is largely involved in inflammation resolution and tissue healing by secreting cytokins such as IL-10, IL-4, etc, counter acting the deleterious effects of the M1 phenotype (Mantovani et al., 2004). The ability of macrophages to swith back and forth between

the M1 and M2 phenotypes is largely dependent on their enviornment. M1, pro-inflammatory, macrophages are the predominant type seen in periodontitis models in mice, non human primates, and human samples (Gonzalez et al., 2015; Lam et al., 2014; Yang et al., 2017), suggesting its activation contributes to the proinflammatory state during chronic periodontitis.

C-C motif chemokine ligand 2 (CCL2) is a well-known macrophage chemoattractant with the ability to induce polarization from the M1 phenotype to the M2 phenotype (Roca et al., 2009). Recent studies have exhibited the ability of CCL2 to successfully induce macrophage polarization towards the M2 phenotype in the murine periodontitis model resulting in protection against periodontitis induced alveolar bone loss (Zhuang et al., 2019). In our study, we examined the effects of CCL2 administration in a canine ligature induced periodontitis model. CCL2 was administed directly into the gingival sulcus at weeks 0, 4, and 8 to promote a shift in the macrophage population away from the pro-inflammatory M1 phenotype and towards the anti-inflammatory M2. Clinical outcomes were measured in the form of bleeding on probing, change in probing depth (compared to baseline) and micro-CT analysis. We hypothesized that CCL2 host modulation therapy would reduce attachment loss in the canine ligature induced periodontitis model.

Bleeding on probing was measured by recording the presence of blood after probe insertion into the gingival sulcus of P4 and M1. Six points on each tooth were evaluated, straight buccal (SB), mesiobuccal (MB), distobuccal (DB), mesiolingual (ML) distolingual (DL), and straight lingual (SL) at baseline (week 0), week 4, week 8 and week 12. As anticipated, the ligatures created significant inflammation and bleeding on probing in the untreated, blank MP and rhCCL2 MP groups compared to healthy control, which had no ligatures. The rhCCL2 MPs failed to show a reduction in bleeding on probing compared to the untreated group at any of the times recorded.

However, during data collection it was very difficult to decern sites without bleeding on probing due to the extensive inflammation present. Visual inspection of the dogs did show less redness and edema in the rhCCL2 MP dogs compared to the untreated and blank MPs (Figure 2), however these data sets are difficult to standardize.

Probing depths represented the distance from free gingival margin to the base of the sulcus, taken in the same six sites as bleeding on probing: straight buccal (SB), mesiobuccal (MB), distobuccal (DB), mesiolingual (ML) distolingual (DL), and straight lingual (SL). Change in probing depth was calculated by subtracting the probing depth at weeks 4, 8, and 12 from baseline (week 0) at each individual site. At the conclusion of the study (week 12), the rhCCL2 MP group showed an average probing depth change of 0.53mm versus 0.50mm in the healthy control group. Both the rhCCL2 MP and healthy control groups exhibited significantly less bone loss compared to the untreated and blank MP groups (1.05mm & 1.23mm). Based on these results we can conclude that rhCCL2 MPs successfully prevented alveolar bone loss compared to the untreated and blank MP groups. Interestingly, rhCCL2 MPs provided this protection against alveolar bone loss under conditions of heavy inflammation and bleeding on probing. We can infer from this data that rhCCL2 MPs were able to minimize osteoclastic activity equivalent to the healthy controls, without reducing the level of inflammation and bleeding on probing.

At week 12, the canines were sacrificed and micro-CT analysis was performed on their mandibles to evaluate the level of alveolar bone loss. The micro-CT analysis revealed 0.74mm of bone loss (from the CEJ-ABC) in the untreated group, compared to 0.52mm in the rhCCL2 MP group and 0.35mm in the healthy control group. The rhCCL2 MP group showed less alveolar bone loss compared to the untreated group and was statistically closer to the healthy control (P value of 0.0739) than to the untreated group (P value of 0.0544) which was trending towards

statistical significance. Because this was not a split mouth design study, there were no real baseline value to help standardize the data. It is likely that the canines all started with varying levels of bone loss at the initiation of the study and since micro-CT analysis. One possible way to minimize this variance in the future is to increase the number of subjects in the study. In all, the administration of rh CCL2 MPs in the presence of ligature induced periodontitis in the canine model showed protection against attachment loss, measured by changes in probing depth and alveolar bone loss. CCL2 was able to accomplish this even in the presence of significant inflammation and bleeding on probing.

Interestingly, there are several *in vivo* experimental studies that support host modulation therapy as a successful approach to treating periodontal disease, targeting various components of the inflammatory response. Whereas CCL2 promotes macrophage phenotype conversion from pro-inflammatory to anti-inflammatory, C-C motif chemokine ligand 22 (CCL22) promotes accumulation of regulatory T cells (Tregs) shifting the local inflammatory environment towards homeostasis. Glowacki et al evaluated the efficacy of CCL22 administered locally to the periodontium in the murine and canine model on the progression of ligature induced periodontal disease (Glowacki et al., 2013). In these animal models, CCL22 was able to recruit Tregs and significantly decrease the progression of periodontitis resulting in less alveolar bone loss (murine and canine) and improved clinical scores of disease progression (canine). Moreover, the Treg recruitment resulted in significant decreases in proinflammatory cytokines and increase in anti-inflammatory cytokines in the periodontal tissues without reducing the bacterial load.

Specialized pro-resolvin mediators (SPMs) are a family of signal carriers that play an active role in the resolution of acute inflammation and represent another target for host modulation therapy in the treatment of periodontitis. Resolvin E1 (RvE1) is a specialized pro-resolvin

mediator that effectively treated *P.gingivalis* induced periodontitis in rabbits via local administration to the marginal gingival tissue over a 6-week period. RvE1 promoted the resolution of inflammation and resulted in restoration of the local lesion, and complete regeneration of diseased tissue, including bone (Hatice Hasturk et al., 2007). This provides yet another example of how HMT is a promising avenue of disease treatment by reversing the destructive inflammatory process initiated by periodontal pathogens.

Future applications of a host modulatory cytokine like rhCCL2 are very promising, especially in human subjects. NHANES studies from 2009 to 2012 estimated that 46% of US dentate adults aged 30 years (representing about 141.0 million adults) had periodontitis, with 8.9% having severe periodontitis and 37.1% having less severe periodontitis (Eke et al., 2015). Additionally, 88% of the population presented with attachment loss of greater than 3mm on at least one site, while 42% presented with probing depths of greater than 4mm on at least one site. We also know from previous epidemiological studies that about 8% of the population fall in the "rapid progression" group, which is categorized by an average of 9mm of attachment loss by the age of 35 (Loe, Anerud, Boysen, & Morrison, 1986). Evidence suggests that even with frequent periodontal treatment over time, about 3-4.2% of the population are considered "extreme downhill" patients losing greater than 10 teeth over the duration of their lives (Hirschfeld & Wasserman, 1978; McFall, 1982). The ability of rhCCL2 to alter the macrophage phenotype in the presence of inflammation is an exciting prospect for many of these patients that do not respond well to conventional periodontal therapy. The field of host modulation therapy will continue to expand and evolve and our hope is that rhCCL2 will eventually yield promising results in human subjects.

List of Tables

#1: Treatment Groups

Table 1

Group Number	Ligatures (Y/N)	Treatment Modality	Number of Participants (N)
#1	N	Healthy Control	N = 24
#2	Y	Untreated	N= 48
#3	Y	Blank microparticles (MPs)	N = 24
#4	Y	rhCCL2 microparticles (MPs)	N = 48

List of Figures

Figure #1: Study Design

Figure 1

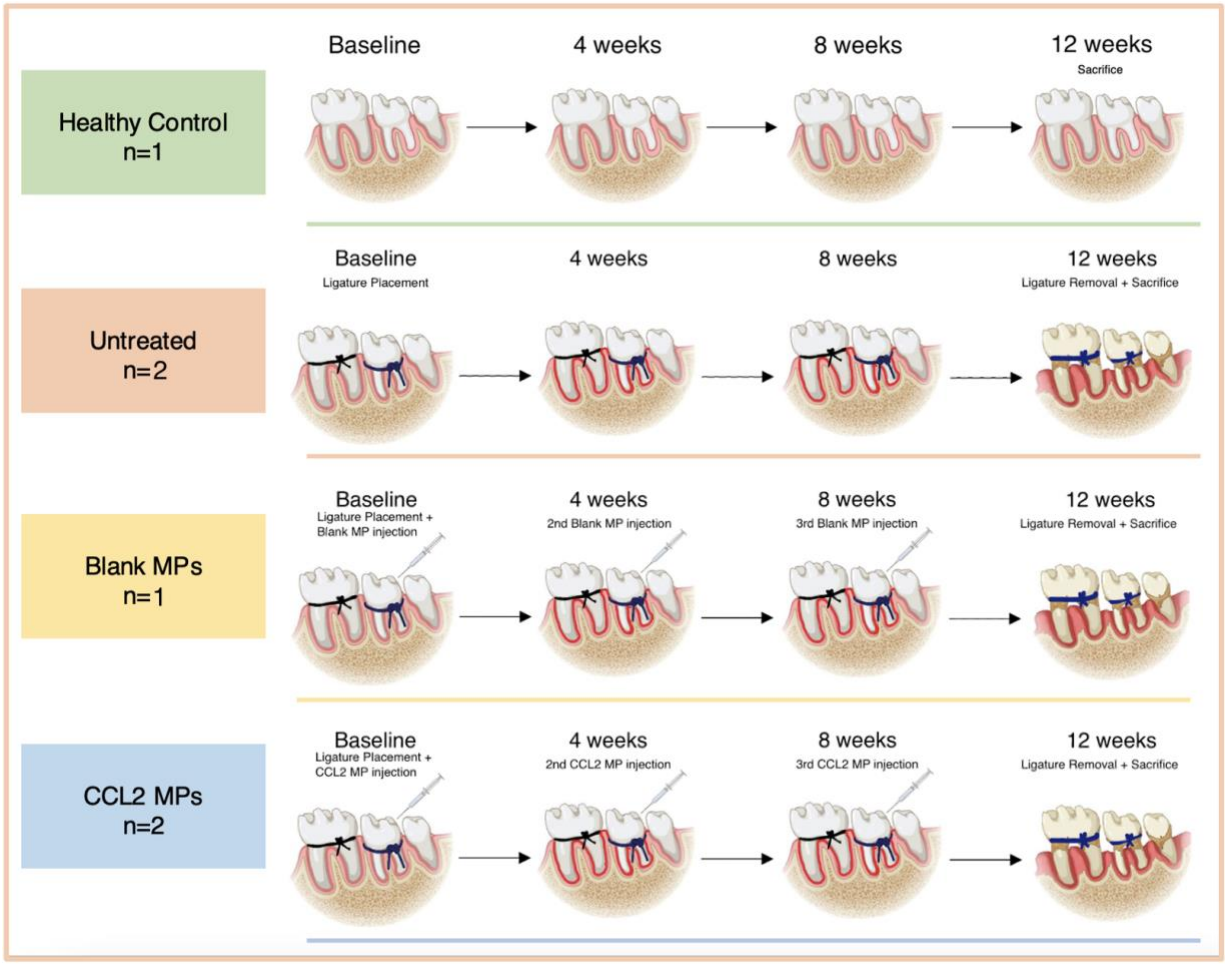
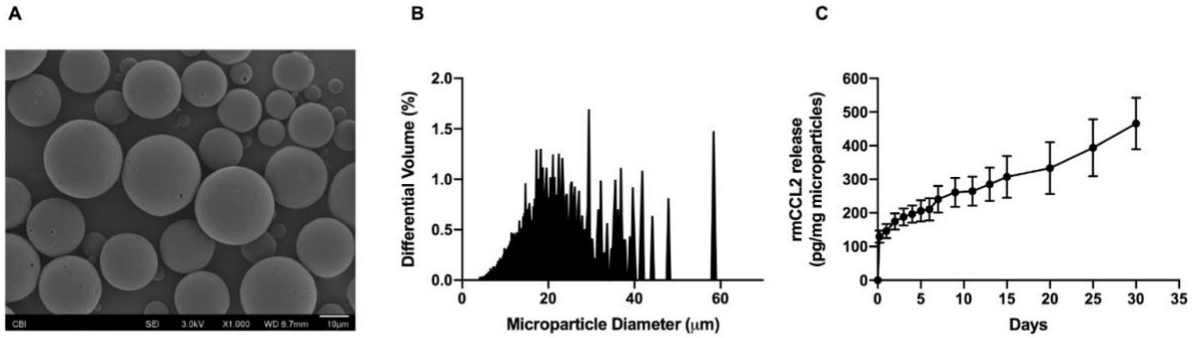


Figure #2: CCL2 PLGA MP Characterization

Figure 2



CCL2 PLGA MP characterization, size distribution and release profile assessment

Figure #3: Release Profile of rhCCL2 MPs

Figure 3

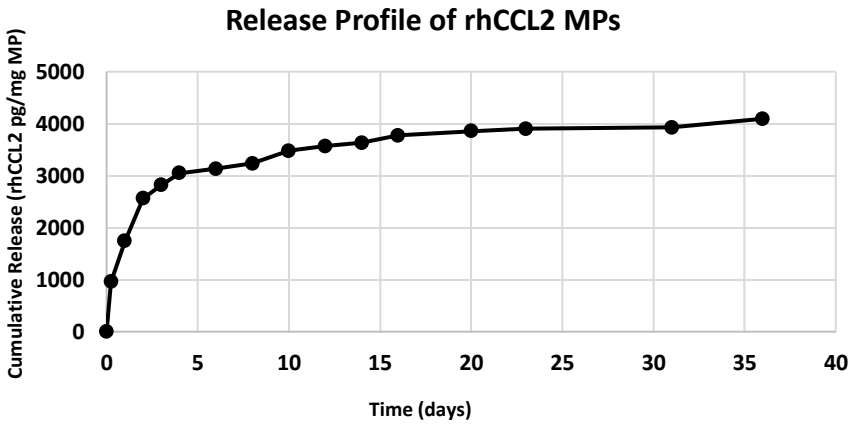


Figure #4: Clinical Photographs of BOP at Week 12

Figure 4

Figure #5: Results of BOP at Weeks 0, 4, 8, & 12

Figure 5

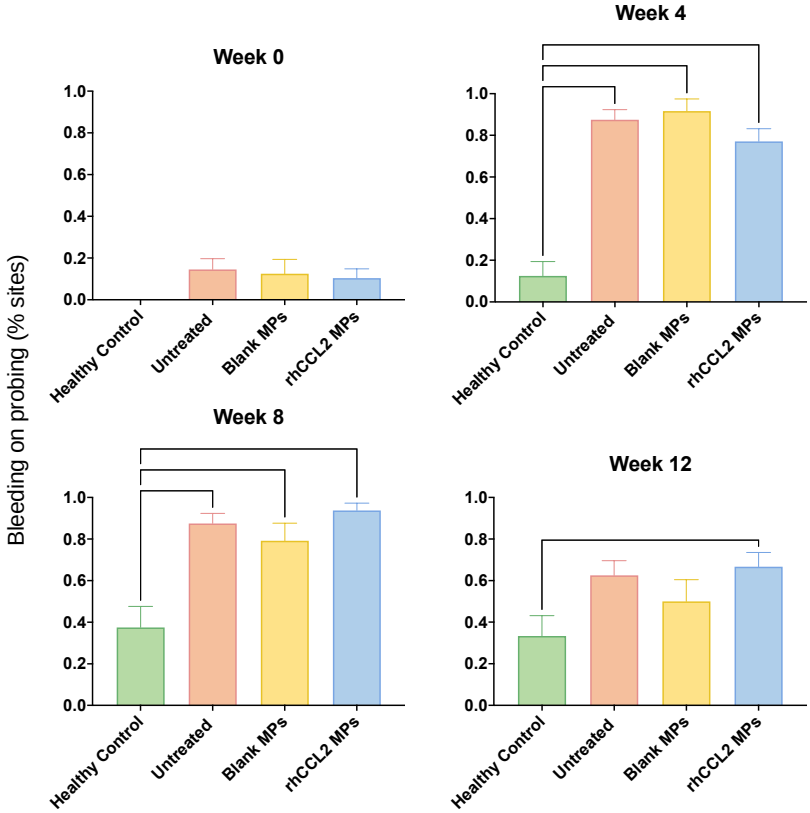


Figure #6: Results of Change in Probing Depths at Weeks 4, 8, 12

Figure 6

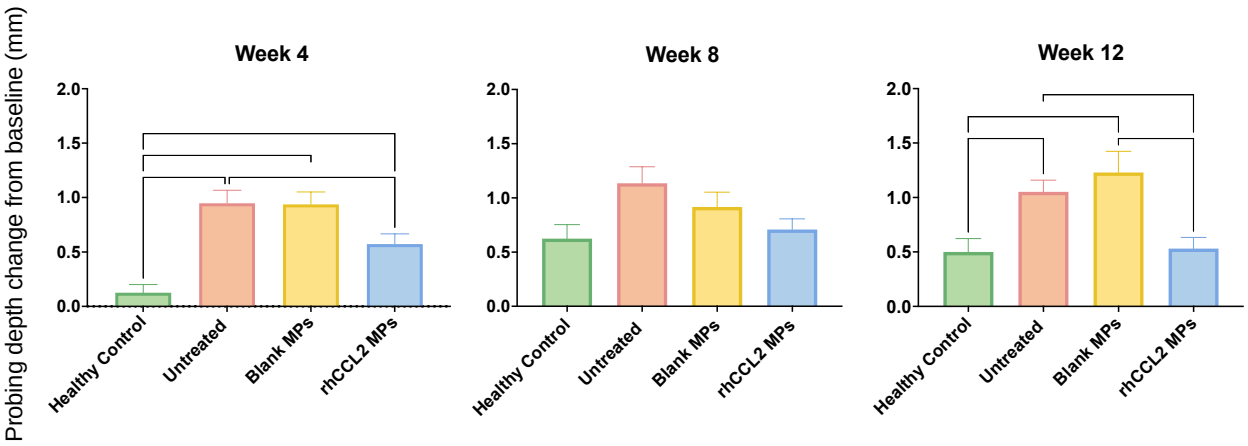
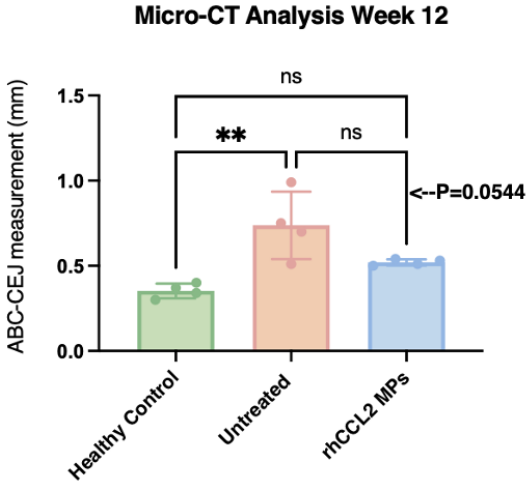


Figure #7: Results of Micro-CT Analysis at Week 12

Figure 7



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