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Cannabidiol alters the immune response to *Porphyromonas gingivalis* in mice.

Shilpa Singh
University of Louisville

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CANNABIDIOL ALTERS THE IMMUNE RESPONSE TO
PORPHYROMONAS GINGIVALIS IN MICE

By

Shilpa Singh

A Thesis
Submitted to the Faculty of the
School of Dentistry of the University of Louisville
In Partial Fulfillment of the Requirement for the Degree of

Master of Science in Oral Biology

Department of Oral Immunology and Infectious Diseases
University of Louisville School of Dentistry
Louisville, KY

August 2018

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GINGIVALIS INFECTION IN MICE

By

Shilpa Singh

A Thesis approved on

August 6, 2018

By the following Thesis Committee:

Dr. David A. Scott (Mentor)

Dr. Huizhi Wang (Co/mepvt)

Dr. Juhi Bagaitkar (Committee member)

DEDICATION

To my husband,

Aarohan Singh

for his never-ending support and patience.

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Foremost, my deepest gratitude goes to my mentor, Dr. David A Scott for patiently guiding me and encouraging me throughout the duration of this research study. I cannot thank Dr. Scott enough for his never-ending confidence in me and helping me realize my capabilities that will always have an impact in my future.

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ABSTRACT

CANNABIDIOL ALTERS THE IMMUNE RESPONSE TO *PORPHYROMONAS GINGIVALIS* IN MICE

Shilpa Singh

August 6, 2018

Background: *Porphyromonas gingivalis* is an important etiologic agent of chronic periodontitis, an infectious disease defined by destruction of supporting tissues of the teeth. Marijuana is a risk factor for chronic periodontitis, although underlying mechanisms are poorly understood. As phytocannabinoids have been ascribed anti-inflammatory properties, we hypothesized that cannabidiol exposure would lead to altered immune response to *P. gingivalis* infection facilitating bacterial persistence and, subsequently, increased alveolar bone loss in cannabidiol (CBD)-exposed mice.

Methods: *P. gingivalis*-infected cannabinoid receptor 2 (CB2) knockout and wild type C57/Bl6 mice were exposed, or not exposed, to cannabidiol. Oral swabs from mice were used to monitor the presence of *P. gingivalis* by PCR and culture. Skulls were harvested, defleshed and stained to analyze the amount of bone loss under microscope. Alveolar bone volume and alveolar bone density were also analyzed by using μ CT. Systemic *P. gingivalis*-specific and total IgG and IgM antibodies were quantified by ELISA. Periodontal expression of inflammatory markers – CD14, CD45, MIP-2, MMP-9 and

IL-1 β was measured in the maxillary gingivae by qPCR.

Results: In our hands oral gavage model appears to be one of repeated transient infection with *P. gingivalis* rather than a persistent colonization model. Although *P. gingivalis* could not be detected in oral swabs, the infection was sufficient to produce an immune reaction in gingiva and serum. Cannabidiol suppresses total IgM production in mice. If this occurs in humans, it applies that adaptive immune response is less well equipped for the arrival of oral pathogens. However, CBD did not affect *P. gingivalis* specific IgG response. CBD significantly reduced the expression of IL-1 β , CD45 and CD14 inflammatory markers in maxillary gingiva in *P. gingivalis* infected wild type mice but not CB2^{-/-} mice. Deficient immune response to *P. gingivalis* may lead to its persistence and more severe periodontal destruction in cannabis users. This is analogous with *in-vitro* nicotine studies and with clinical studies showing more severe clinical manifestations of chronic periodontitis in tobacco smokers. However, our results showed a reduction in MMP-9 expression in presence of cannabidiol. This anomaly is hard to explain and does not correspond with the findings in cigarette smokers where MMP-9 levels are significantly higher. Increased bone loss was hypothesized because we know cannabis drives bone loss in animals and humans. This did not happen here. May be because we didn't have persistent infection. However, immune differences between wild type versus knock out mice suggest that we could see a long-term effect in chronic infection.

Conclusion and Practical Implications: The results show that cannabidiol alters the immune response of mice infected by *P. gingivalis*. Further studies aimed at understanding the effects of altered immune response in presence of cannabidiol should be performed. This is particularly important as prevalence of cannabis smoking is currently on the rise, partly due to legalization or decriminalization in increasing jurisdictions.

Keywords - Alveolar bone loss, Cannabidiol, CB2, Periodontitis, Phytocannabinoids, *Porphyromonas gingivalis*

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CHAPTER 1: INTRODUCTION

Chronic Periodontitis

Chronic periodontitis predominantly affects adults, but aggressive periodontitis may occasionally occur in children. According to data derived from the National Health and Nutritional Health Examination Survey, around half of Americans above 30 years of age have chronic periodontitis, while 70% of people above 65 years suffer from this disease (Eke et al., 2015). Around 65% of people living under federal poverty level suffer from periodontitis. Chronic periodontitis is a significant public issue since treatment cost is very expensive and affects global adult population (Petersen, Bourgeois, Ogawa, Estupinan-Day, & Ndiaye, 2005).

The term ‘periodontal diseases’ encompasses a wide variety of chronic inflammatory conditions of the gingiva (or gums, the soft tissue surrounding the teeth), bone and ligament (the connective tissue collagen fibers that anchor a tooth to alveolar bone) supporting the teeth (Kinane, Stathopoulou, & Papapanou, 2017). Chronic periodontitis is classified as generalized chronic periodontitis when it affects >10 of the 32 teeth in the human dentition and localized when fewer teeth are involved, less than 10 (Flemmig, 1999).

Chronic periodontitis is characterized by the manifestation of slow irreversible damage of periodontal supporting tissue over time (Agrali & Kuru, 2015). It results in progressive attachment and alveolar bone loss leading to premature tooth loss.

Periodontal disease begins with gingivitis, the localized inflammation of the gingiva that is initiated by bacteria in the dental plaque, which is a microbial biofilm that forms on the teeth and gingiva. Chronic periodontitis occurs when untreated gingivitis progresses to gingival recession, loss of bone and ligament, which creates the deep periodontal 'pockets' that are a hallmark of the disease and can eventually lead to tooth loss.

Although gingivitis and chronic periodontitis are initiated and sustained by the microbial biofilm of the dental plaque, genetic and environmental host factors influence the rate of the disease (Kinane et al., 2017). Sustainance of chronic periodontitis state also depends on host inflammatory reaction to the microbial deposits.

Chronic periodontitis is an infectious disease that occurs because of challenge between host response and a dysbiotic oral microbiome. Periodontal diseases may aggravate the body's overall inflammatory burden, worsening conditions such as diabetes mellitus and atherosclerosis (Khader, Dauod, El-Qaderi, Alkafajei, & Batayha, 2006). Increasing evidence indicates periodontitis to be a risk factor for several other systemic diseases including cardiovascular diseases and adverse pregnancy outcomes (Kinane et al., 2017).

One of the first immune responses to presence of pathogenic bacteria is inflammation. In chronic periodontitis, the pathogen bacteria present in dental plaque lead to activation of host immune responses. Inflammation is stimulated by chemical factors released by injured cells and serves to establish a physical barrier against the spread of infection, and to promote healing of any damaged tissue following the clearance of pathogens.

Inflammatory markers released from immune cells play an important role in initiation of innate immune responses, ensuring clearance of pathogen (Stvrtnová, 1995).

CD14 and CD45 are two of the inflammatory markers responsible for initiation of innate host immune responses and clearance of pathogen. CD14 is expressed mainly by macrophages and neutrophils. It acts as a co receptor (along with Toll Like Receptor 4) for detection of bacterial polysaccharide (Kitchens, 2000). CD14 plays a pivotal role in TLR4 activation that in turn has a crucial role in activation of innate immunity and pathogen recognition (Kirschning & Bauer, 2001). CD45 isoforms are present on all nucleated hematopoietic cells. It appears to play a significant role in the ability of immune cells to respond to activating stimuli (Donovan & Koretzky, 1993). CD45 inflammatory marker (leukocyte common antigen) causes increase in cytokine production and proliferation of T cells (Hermiston, Xu, & Weiss, 2003). The inflammatory markers play an important role in the process of clearance of pathogen and hence, resolution of chronic periodontitis

Risk Factors for Chronic Periodontitis

The severity of the periodontal disease is strongly influenced by multiple environmental and host risk factors, both modifiable (for example, smoking) and non-modifiable (for example, genetic susceptibility) (Kinane et al., 2017). Because of general health implications of chronic periodontitis, the CDC has recommended public health programs to control the risk factors (Eke et al., 2016). The CDC is currently working with key partner organizations such as the American Academy of Periodontology and the American Dental Association to improve and sustain surveillance of periodontal disease in the adult U.S. population. Epidemiological studies in the United States have shown that low educational attainment, income below the federal poverty line, Mexican-American ethnicity and African-American ethnicity have all been associated with poor periodontal status in multivariable analyses (Eke, Wei, Thornton-Evans, et al., 2016).

Cigarette smoking is a major modifiable risk factor. Smokers have worse periodontal status and experience more severe tooth loss than non-smokers (Kinane et al., 2017). A study by Zambon JJ et al, examined the association between cigarette smoking and subgingival infection with periodontal pathogens to determine if smokers are more likely to be infected with certain periodontal pathogens than non-smokers. The researchers concluded *Porphyromonas gingivalis* (*P. gingivalis*) was also more likely to subgingivally infect smokers than non-smokers, hence increasing the risk for occurrence of chronic periodontitis (Zambon et al., 1996). Tobacco smoke profoundly influences the biological mediators that control bleeding responses to bacterial challenge (Dietrich,

Bernimoulin, & Glynn, 2004), promotes oral bacterial dysbiosis (Kumar, 2012), compromises innate cell function (Palmer, Wilson, Hasan, & Scott, 2005) and promotes protease-antiprotease imbalance (Buduneli & Scott, 2018).

Diabetes mellitus is an established risk factor for periodontitis. Mechanistic studies indicate that diabetes mellitus leads to a hyperinflammatory response to the periodontal microbiota and impairs resolution of inflammation and repair, which leads to accelerated periodontal destruction (Lalla & Papapanou, 2011). In a study, individuals those who were long-duration diabetics, especially those over 30 years old, demonstrated more attachment loss, bone loss, and deeper probing pocket depths than their nondiabetic controls (Grant-Theule, 1996).

Alterations in the host inflammatory response may have a major influence on the increased prevalence and severity of periodontal destruction seen in diabetes (Mealey & Oates, 2006). The function of immune cells, including neutrophils, monocytes and macrophages, is altered in diabetes ("Position Paper: Diabetes and Periodontal Diseases," 2000). Neutrophil adherence, chemotaxis, and phagocytosis are often impaired, which may inhibit bacterial killing in the periodontal pocket and significantly increase periodontal destruction (Mealey & Oates, 2006). Impaired host immune response to the periodontal pathogenic bacteria lead to increased occurrence of periodontitis in diabetic patients (Grant-Theule, 1996).

The role of genetics in chronic periodontitis has been investigated in family and twin studies. A study in young Indonesian siblings who did not develop severe chronic periodontitis despite not receiving regular dental care suggested that genetic factors could underlie the less-severe forms of periodontal disease (Pillet, Pozzetto, & Roblin, 2016). Intensive research is underway to identify the genes and polymorphisms associated with all forms of periodontal disease. Numerous genes are involved in chronic periodontitis, and the genotypes of chronic periodontitis might vary across individuals and ethnicities. Genetic defects in the recognition and response pathways of the host to identify microbial pathogens predispose to either altered microbial colonization or misrecognition of normal microbiota leading to dysbiosis and appearance of infectious disease (Nibali, Henderson, Sadiq, & Donos, 2014).

Pathogenic bacteria in the biofilm or dental plaque are strongly associated with pathogenesis of periodontal diseases. Disease occurs when the balance between the microbial biofilm and the host is lost, owing to dysbiosis accompanied with an immune overreaction to microbial presence (Olsen et al., 2017).

Cannabis – A Risk Factor for Chronic Periodontitis

Cannabis is a major emerging risk factor for chronic periodontitis. Several human studies indicate increased alveolar bone loss in people using cannabis. A ‘cohort study’ by Meier et al (2016), showed association between cannabis and periodontal disease in humans. Shariff JA (2016), analyzed the data from National Health and Nutrition Examination Survey (2011-2012) and showed that frequent recreational cannabis use is associated with deeper pocket depths, more clinical attachment loss and higher chances of severe periodontitis. Similarly, a cohort study on humans by Brett et al (2014) reported higher attachment loss in cannabis smokers. Thomson et al (2008) reported strong association of cannabis use with prevalence and incidence of periodontal attachment loss in a prospective ‘cohort study’ of young adults. More human studies (example - Zeng et al (2014), Jamieson et al (2010)) show similar results.

Cannabis use has been associated with poor oral health, higher caries rates, and higher plaque scores. Xerostomia and cannabis stomatitis are reported in frequent cannabis users. Cannabis smoking is reported to be associated with a higher incidence of premalignant lesions and malignant lesions, including head and neck cancer (Momen-Heravi & Kang, 2017).

Although the studies above reported increased risk of periodontitis with the use of cannabis, the underlying mechanism is still unclear.

Porphyromonas gingivalis

Among pathogenic bacteria, *P. gingivalis* is an important etiologic agent and is found to be closely associated with severe forms of periodontal disease (Geng, Liu et al. 2017). *P. gingivalis* is a Gram negative, anaerobic and rod-shaped bacterium. It has been termed as a keystone pathogen capable of promoting dysbiosis in the periodontal microbiota in mice. *P. gingivalis* induces chronic periodontitis accompanied by significant alterations in the number and community organization of the oral commensal bacteria.

Even in less quantity, *P. gingivalis* interferes with innate immunity and leads to changes in the counts and composition of the oral commensal microbiota. The resulting dysbiotic microbial community causes disruption of host–microbial homeostasis, leading to inflammatory bone loss (Olsen, Lambris, & Hajishengallis, 2017).

The ability of *P. gingivalis* to manipulate the host response and promote its chronic persistence in the periodontium also benefits from the presence of companion species and *vice versa* in subgingival plaque (Hajishengallis, 2011). *Streptococcus gordonii* is one of the early colonizers that initiate plaque formation and the ability of *P. gingivalis* to specifically recognize and interact with *S. gordonii* is believed to be important for *P. gingivalis* colonization of bacterial plaque (Ng et al., 2016). *P. gingivalis* long fimbriae bind to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on the streptococcal surface, while its short fimbriae specifically recognize surface proteins

on *S. gordonii* and are required for the development of *P. gingivalis* biofilms on streptococcal substrates (Maeda et al., 2004).

P. gingivalis has several virulence factors many of which are related to subversion of the innate immune system. This ability is what often characterizes a successful pathogen, as it tends to disable the overall host response while simultaneously enhancing the pathogenicity of a polymicrobial community (Olsen & Hajishengallis, 2016).

Retention and growth of *P. gingivalis* on oral surfaces are facilitated by adhesins including fimbriae, hemagglutinins, and proteinases. *P. gingivalis* fimbriae are a critical factor for mediation of interaction of this bacterial organism with host tissues. Fimbriae are critically important for bacterial adhesion (Mysak, 2014). *P. gingivalis* has major and minor fimbriae on its cell surface, and both fimbriae contribute to establish the persistent infection and the development of periodontitis.

Lipopolysaccharide (LPS) is the major component of the outer membrane of *P. gingivalis*. This biomolecule is a potent virulence factor in periodontitis (Mysak et al., 2014). LPS consists of three segments with highly variable and conserved regions. They are a phosphorylated glucosamine disaccharide substituted with fatty acids known as lipid A which forms the matrix of the outermost membrane leaflet, a highly variable O-polysaccharide (O-antigen) and a conserved core oligosaccharide that links lipid A to the O-polysaccharide. Lipid A section is the ‘bioactive center’ of LPS, responsible for its endotoxicity. This is due to the specific and highly sensitive recognition of lipid A by host

cells, which subsequently leads to strong immuno-inflammatory response. The LPS stimulates the host inflammatory pathway by interacting with the Toll-like Receptor 4 (TLR 4). *P. gingivalis* grown in high hemin conditions produces predominantly the isoform of LPS with tetra-acylated lipid A structure (*P. gingivalis* LPS_{1435/1449}). In contrast, under low hemin conditions *P. gingivalis* produces the isoform of LPS with penta-acylated lipid A structure (*P. gingivalis* LPS₁₆₉₀). It has been suggested that shifting LPS into tetra-acylated lipid A structure may dampen the TLR4-mediated immuno-inflammatory response of gingival tissues, allowing the pathogen to invade and proliferate in the gingival tissues, thereby leading to progression of periodontal disease (Herath et al., 2013). *P. gingivalis* LPS induces proinflammatory cytokines, such as IL-1 β , IL-6, and IL-8, which induce periodontal tissue destruction (Mysak et al., 2014).

Gingipains from *P. gingivalis* are one of the most prominent virulence factors on periodontal diseases (Nakayama & Ohara, 2017). Working in concert, gingipains cleave not only constituents of periodontal tissue, including the basement membrane structural protein collagen, but also protective host proteins such as antibodies and components of the complement system. The gingipains exhibit dual functionality in the targeting and degradation of complement proteins (Potempa, 2013). *P. gingivalis* produces three cysteine proteases known as gingipains: arginine-gingipain A and B (RgpA and RgpB), and lysine-gingipain (Kgp), which play an important role in processing/maturation of its own cell surface proteins. Gingipains have an adverse effect on healthy tissues via degradation of many human proteins including complement system proteins, cytokines, integrins, and collagen. Gingipains have effects on host cell functions by disturbance of cellular signal

transduction, facilitating long term infection of *P. gingivalis* by its invasion in gingival epithelial cells (Nakayama & Ohara, 2017).

Gingipains of *P. gingivalis* facilitate the evasion of innate immune response of the host by degrading the complement system. The complement system is a major part of the innate immune system and is responsible for the host defense against invading microorganisms. Complement is the first defense line in the host against invading microbes and is a major bridge between innate and adaptive immunity. At high concentrations of gingipains, likely to occur when *P. gingivalis* has established its colonization, the released gingipains can inhibit the bactericidal activity of complement by degrading C3 complement component. This function can prevent opsonization of complement-sensitive bacteria in the proximity of *P. gingivalis*, thereby promoting mixed-species biofilm development.

Zhang and colleagues established that *P. gingivalis* can invade osteoblasts and inhibit their maturation and mineralization in vitro (Zhang, Swearingen, Ju, Rigney, & Tribble, 2010). *P. gingivalis* LPS, lipids and metabolic products can inhibit the differentiation and osteogenesis of osteoblasts and inhibit alveolar bone formation (Zhang, Ju, Rigney, & Tribble, 2014). A study by Zhang et al showed that *P. gingivalis* can invade periodontal cells and disturb the homeostasis of osteoblasts and osteoclasts, which contributes to alveolar bone loss (Zhang et al., 2014).

Hence, *P. gingivalis* prevalence can degrade the innate host responses and cause destruction of tooth supporting tissues directly through number of virulence factors.

Cannabis and Legal Status

As of January 22, 2018, 29 states, the District of Columbia, Guam and Puerto Rico have legalized cannabis use for medical purposes (State Medical Marijuana Laws).

While, seven states and the District of Columbia have legalized the recreational use of cannabis. As more states consider legalizing medical or recreational use of cannabis, determining the effects of such laws on usage patterns and related outcomes have become an important public health priority (Carliner, Brown, Sarvet, & Hasin, 2017).

Among adults, cannabis use has increased considerably over the last 15 years, as have adverse health consequences of use, including potential harms to a developing fetus, alterations to neurodevelopment when used early in adolescence, risks for a withdrawal syndrome and for cannabis use disorder, accidents and fatalities, and impairment in many social domains, including education and employment. Among adults, cannabis use is generally increasing, and increased prevalence of cannabis use over time has also been observed among diverse sociodemographic groups (Carliner et al., 2017).

The medical use of cannabis products to treat disease or improve symptoms remains debated, even if the benefits and risks of such medications have been investigated in clinical trials on multiple sclerosis, cancer and non-cancer pain, neurodegenerative disorders, and appetite suppression (Whiting et al., 2015).

Apart from the legal use of cannabis, the National Institute on Drug Abuse reported that cannabis is the most commonly used illegal drug in the United States (2015 National Survey on Drug Use and Health). Recreational use has grown and appears to be associated with serious mental and physical health problems such as suicidal thoughts and rapid heart rate (Debruyne & Le Boisselier, 2015). Cannabis is globally the most widely used illicit drug, the World Health Organization estimating that 181.1 million people ages 15-64 years used cannabis for nonmedical purposes in the year 2013 (Le Boisselier, Alexandre, Lelong-Boulouard, & Debruyne, 2017).

Owing to this increased consumption of cannabis, it is imperative to understand the relationship of medical and recreational marijuana laws to adult health outcomes. This study focusses on the relationship between cannabis use and alveolar bone loss.

Phytocannabinoids

Cannabis, also known as marijuana, is the crushed dried leaves and flowers obtained from *Cannabis sativa* and *Cannabis indica* plants and contains naturally occurring phytocannabinoids. The cannabis flower is comprised of over 100 different cannabinoids, the active compounds found within the cannabis plant. Cannabinoids are a collective group of compounds that act on cannabinoid receptors. They include plant-derived phytocannabinoids, synthetic cannabinoids, and endogenously-derived endocannabinoids.

Cannabinoids work on the endocannabinoid system (ECS) which consists of a series of neuromodulatory lipids and receptors located throughout the brain and central and peripheral nervous system, which accept endogenous cannabinoids (anandamide, 2-arachidonoylglycerol) and phytocannabinoids (plant-based) (Babson, Sottile, & Morabito, 2017).

Cannabinoid receptors, CB1 receptors and CB2 receptors are two main receptors within the ECS. The cannabinoid receptors are members of the superfamily of G-protein-coupled receptors.

CB1 receptor is most abundant at central and peripheral nerve terminals where its activation mediates inhibition of neurotransmitter release. CB1 receptors are mainly involved in the central effects of cannabinoids, which include consequences on learning,

memory cognition, emotion, movement, sensory perception, and nausea, as well as the psychoactive properties associated with cannabinoids (Kelly & Nappe, 2018).

CB2 receptor is predominantly found on immune and inflammatory cells and is likely involved in the regulation of both cytokine release and chemotaxis. CB2 receptors affect inflammation and immune system regulation (Kelly & Nappe, 2018).

The two most studied endogenous cannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), are synthesized from lipid precursors. Endocannabinoids bind to CB1R and CB2R with AEA and 2-AG binding preferentially to CB1R and CB2R, respectively. The endogenous cannabinoids, together with their receptors and the enzymes involved in their metabolism, form the ECS (Barutta et al., 2018). Endocannabinoids may potentially mediate or modulate numerous neurophysiological processes including nociception. When activated, these neurotransmitters seem to aid in the regulation of several functions, including coordination, pain perception, cognition, gastrointestinal motility, heart rate, immune function, and intraocular pressure (Vadivelu, Kai, Kodumudi, Sramcik, & Kaye, 2018).

The major phytocannabinoids are delta-9 tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN) (Barutta, Mastrocola, Bellini, Bruno, & Gruden, 2018).

THC is the primary psychoactive component of cannabis which is responsible for the “high” associated with cannabis use. THC acts on CB1 receptors and yields a biphasic

effect such that the impact of THC varies between low and high doses. Because of its psychoactive effects, THC is used for recreational purposes.

CBN is the primary product of THC degradation, and there is usually little of it in a fresh plant. CBN content increases as THC degrades in storage, and with exposure to light and air. It is only mildly psychoactive. Its affinity to the CB2R is higher than for the CB1R (Mahadevan et al., 2000).

Cannabinoids and bone health

Mice with CB1 receptor deficiency have high peak bone mass because of an osteoclast defect and develop age-related osteoporosis because of impaired bone formation and accumulation of bone marrow fat. Mice with CB2 deficiency have relatively normal peak bone mass but develop age-related osteoporosis because of increased bone turnover with uncoupling of bone resorption from bone formation. Mice with GPR55 deficiency have increased bone mass because of a defect in the resorptive activity of osteoclasts, but bone formation is unaffected (Idris et al., 2005).

According to a cross sectional study on humans, heavy cannabis users had reduced bone density as compared to controls as well as reduced body mass index, high bone turnover, and an increase rate of fractures (Sophocleous et al., 2017).

A study performed by Wasserman et al showed femur of mice lacking CB1 or CB2 were considerably longer at the end of the rapid growth phase compared to wild-type animals. Importantly, THC slowed skeletal elongation of wild type and CB2 knockouts, but not that of CB1 deficient mice. This was probably due to a direct effect of THC on hypertrophic chondrocytes, because THC inhibited EGC chondrocyte hypertrophy in ex vivo cultures and reduced the hypertrophic cell zone thickness of treated animals (Wasserman et al., 2015). These experimental findings are in line with human studies, reporting that babies born to marijuana using mothers have a reduced fetal growth rate, resulting in a reduced

birth weight, a shorter stature and a reduced head size at birth ((El Marroun et al., 2009; Zuckerman et al., 1989).

Cannabidiol (CBD) and Immunity

CBD is a non-intoxicating constituent of cannabis which acts on CB2R. CBD has been shown to counter the effects of THC. Owing to its anti-inflammatory effects, CBD has received a lot of attention as a potential therapeutic (Babson et al., 2017).

Strong experimental evidence exists for a role for the endocannabinoid system, in particular CB2, in immune modulation. The CB2R is expressed by all immune cells.

In humans, the highest to lowest CB2-expressing immune cells are: B cells > NK cells > macrophages > polymorphonuclear cells > CD4 T cells > CD8 T cells (Galiegue et al., 1995).

CB2 has been found to play a complex role in regulating migration, proliferation and different effector functions of various immune cells (Basu & Dittel, 2011). Both in vitro and in vivo studies indicate that CB2 can suppress immune responses.

Chronic Periodontitis and Immunity

Chronic Periodontitis arises from complex interactions between the host and the subgingival dysbiotic microbiota that lead to excessive or dysregulated inflammatory responses involving elements of both innate (complement and phagocytes) and adaptive (regulatory and effector lymphocytes) immunity (Hajishengallis & Korostoff, 2017). Variations in the host response may increase or decrease the susceptibility of different individuals to destructive periodontal disease (Benakanakere, Abdolhosseini, Hosur, Finoti, & Kinane, 2015).

The host–pathogen interactions that occur at the gingival crevice and periodontal pocket site are characterized by neutrophil and granulocyte (polymorphonuclear cell) infiltration, which is driven by chemotactic gradients created by the bacteria and the inflammatory response and lymphocyte infiltration. The resulting pro-inflammatory agents include cytokines such as tumor necrosis factor (TNF), interleukins, interferon- γ (IFN γ), as well as antibodies raised against the biofilm components (Kinane et al., 2017).

Epithelial cells function as a physical barrier against pathogens and elicit innate immune responses. Dendritic Langerhans cells within the epithelium take up microbial antigenic material and bring it to the lymphoid tissue for presentation to lymphocytes. The periodontal lesion is infiltrated with neutrophils, granulocyte (neutrophils are granulocytes) and lymphocytes. Neutrophils attempt to engulf and kill bacteria but are overwhelmed by the magnitude and chronic persistence of the microbial biofilm. This severe chronic

inflammatory response leads to alveolar bone resorption by osteoclasts, and degradation of ligament fibers by matrix metalloproteinases and the formation of granulation tissue (Sorsa et al., 2016). This pathophysiological situation persists until the tooth is exfoliated or the microbial biofilm and granulation tissue are successfully removed therapeutically. Thus, host immune response plays an important role in clearance of pathogen and thus in resolution of the inflammatory response.

Cannabinoids and Chronic Periodontitis

The pathogenesis of chronic periodontitis begins with bacterial accumulation on the hard and soft tissues surrounding the tooth (Pihlstrom, Michalowicz, & Johnson, 2005). To deal with the vast array of the continuous microbial challenge present in the supra and subgingival biofilm, the immune system confers a protective immune response to fight off invading pathogens. However, destructive immune response can occur when that challenge overwhelms the host or is dysregulated during immune reaction (Teng, 2006). Cannabinoid receptors, particularly CB2 receptors are present on all immune cells, indicating that they can modulate the host immune response in the presence of infection (Basu & Dittel, 2011).

The ability of immune cells to migrate throughout the body is critical for their development/maturation as well as for the induction of the immune response during infection. CB2 showed a complex regulation pattern with some CB2-ligands promoting and others inhibiting chemotaxis. In-vitro studies showed CB2-dependent immune cell migration in response to 2-AG and that synthetic CB2-agonists, at least in some circumstances, has the capacity to inhibit migration to external stimuli (Basu & Dittel, 2011). Thus, CBD that acts on CB2 receptor may reduce the immune cell migrations and affect the innate host responses in presence of infection. The suppressed innate host response may lead to persistence of subgingival pathogenic bacteria leading to chronic periodontitis.

Cytokines are a large group of proteins that include interleukins (IL), interferons (IFN), and growth factors that are produced and secreted by immune cells and play key roles in coordinating appropriate immune responses for the resolution of chronic periodontitis (Gemmell, Marshall, & Seymour, 1997). The balance between pro-inflammatory and anti-inflammatory cytokines is crucial for the resolution of the inflammatory reaction in chronic periodontitis. Some studies support a role for CB2 in immunosuppression by inhibiting the production of pro-inflammatory cytokines and perhaps by enhancing anti-inflammatory cytokines ((Correa et al., 2010), (Correa et al., 2009)). Hence, presence of cannabidiol may lead to progression of chronic periodontitis in absence of balanced action of cytokines that aid in innate host response.

Further, according to an *in-vivo* study, CB2 receptor deficient mice had significantly lower bacterial loads and a significantly higher survival rate as compared to wild type mice. This was correlated with lower levels of both the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokine IL-6 (Csoka et al., 2009). This suggests that CB2 restricts the immune response required for bacterial clearance allowing for the development of a detrimental inflammatory response. Persistence of pathogenic bacteria in the oral cavity may result in exacerbated periodontal destruction through direct virulence factors.

Some literature studies ((Ossola, Surkin, Mohn, Elverdin, & Fernandez-Solari, 2016), (Napimoga et al., 2009)) indicate that cannabinoids can be used to modulate the host response and reduce the severity of chronic periodontitis. The basis of this assumption is that chronic periodontitis is marked by the presence of pro-inflammatory cytokines and

chemokines such as, interleukin 1 beta (IL-1 β), interleukin-6 (IL-6), interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1). These inflammatory mediators propagate the inflammation leading to bone resorption and destruction of periodontal tissue (Graves, Oates, & Garlet, 2011). One such study by Abidi et al suggests that CB2 receptor ligand agonist downregulated *P. gingivalis* LPS, IL-1 β , and TNF- α stimulated IL-6 and MCP-1 levels in primary human periodontal ligament fibroblasts. According to this study, the downregulation of inflammatory mediators may be beneficial in treating chronic periodontitis (Abidi et al., 2018). To monitor host immune response in presence of cannabidiol, we monitored the expression of inflammatory markers – CD14, CD45, MMP-9, MIP-2 and IL-1 β .

Mice Periodontal Disease Model

Animal models and cell cultures have contributed new knowledge in biological sciences, including periodontology. Although cultured cells can be used to study physiological processes that occur during the pathogenesis of periodontitis, the complex host response fundamentally responsible for this disease cannot be reproduced *in vitro* (Helieh, 2011). Experimental animal models are critical tools to investigate mechanisms of periodontal pathogenesis and test new therapeutic approaches. Mice constitute the most convenient and versatile model for mechanistic immunological research owing to availability of genetically defined strains that display specific immunological abnormalities. Hence, mice periodontal disease models are used to assess the host response and its effects on the tooth-supporting tissues (gingiva and bone) under well-controlled conditions (Abe & Hajishengallis, 2013).

Mouse models of periodontitis have been productively used to investigate periodontal disease mechanisms and to test the potential of novel therapeutic compounds (Baker, 1994). The ligature model is one of the methods used to induce alveolar bone loss in mice. To induce bone loss, a silk ligature is tied typically around maxillary posterior teeth (Abe & Hajishengallis, 2013). An important advantage of the ligature-induced periodontitis model is that disease can be initiated at a known time with a predictable sequence of events culminating in alveolar bone loss within a few days (Bezerra et al., 2000). However, in small animals such as mice, the possibility for mechanical trauma by the ligatures, which could contribute to bone loss cannot be excluded (Abe & Hajishengallis, 2013).

A murine model was developed in which mice are orally infected with *P. gingivalis*, which results in alveolar bone loss (Baker, Evans, & Roopenian, 1994). This model offers many benefits over human studies, such as controlled environmental conditions and infection levels (Hart et al., 2004). Baker et al. used this system to investigate the genetic control of susceptibility to *P. gingivalis*-induced alveolar bone loss (Baker, Dixon, & Roopenian, 2000). This model offers the means to determine how genetic variation can influence the differential host response to oral infection with *P. gingivalis*.

Hence, The Baker mouse model of periodontitis has been used to measure alveolar bone resorption caused by oral bacterial inoculums as an outcome for the clinical presentation of periodontitis in humans (Baker et al., 1994). It was speculated that *P. gingivalis* initiates experimental periodontitis, at least in part, by modifying the endogenous subgingival biofilm to acquire enhanced virulence (Kinane & Hajishengallis, 2009). In this model, mice naturally develop periodontitis with further increases as a function of age, similar to human periodontitis (Helieh, 2011). Therefore, this model is widely used to detect bone loss levels in mice after being exposed to *P. gingivalis* infection.

Summary

As discussed above, literature studies suggest that CB2 receptor is responsible for downregulating the expression the pro-inflammatory cytokines and enhancing the expression of anti-inflammatory cytokines in the inflammatory process of chronic periodontitis. This may suggest that cannabinoids may have a protective role in chronic periodontitis by inhibiting the inflammatory process. However, ineffective innate immune host response to the pathogenic bacteria like *P. gingivalis* may lead to their persistence in the host tissues. *P. gingivalis*, through its direct virulence factors may lead to destruction of periodontal ligament and further reduce the host immune response through its direct virulence factors as discussed above. Furthermore, *P. gingivalis* being determined as keystone pathogen in murine model, can convert commensal bacteria to pathogenic strains through dysbiosis. This may lead to severe symptoms of chronic periodontitis.

Additionally, this phenomenon can be compared to that seen in smokers who manifest higher periodontal bone loss even in absence of overt inflammatory signs clinically (Preber & Bergstrom, 1986). Like cannabinoids, smoking also impacts on many components of the inflammatory response and on both the innate and adaptive immune responses, which results in worse periodontal destruction in them as compared to non-smokers (Knight, Liu, Seymour, Faggion, & Cullinan, 2016). The higher level of matrix metalloproteinases in smokers than in non-smokers contribute to the worse periodontal tissue destruction (Victor, Subramanian, Gnana, & Kolagani, 2014). Similar mechanism may be involved in higher periodontal tissue destruction in presence of cannabinoids

Hypothesis

Based on the previous human studies, we hypothesized that cannabidiol (CBD) exposure would alter immune response in mice infected with *Porphyromonas gingivalis* and increase the alveolar bone loss. We set out to test this hypothesis in CB2 receptor deficient and control mice.

CHAPTER 2: MATERIALS AND METHODS

Porphyromonas gingivalis ATCC 33277 was purchased from the American Type Culture Collection (Manassas, VA) and maintained as frozen stocks. *P. gingivalis* was grown in Gifu anaerobic medium (GAM; Nissui Pharmaceutical, Tokyo, Japan). *P. gingivalis* was grown under anaerobic conditions (80% N₂, 10% H₂, and 10% CO₂) at 37°C. Growth was monitored by tracking optical density at a wavelength of 600 nm. Cannabidiol dissolved in methanol was obtained from CAYMAN Chemical (Ann Arbor MI, USA). Methanol and Carboxymethyl cellulose (CMC) were purchased from Sigma Aldrich Corporation (St. Louis MO, USA). Phosphate Buffered Saline (PBS) was purchased from Life Technologies (Grand Island NY, USA). Isoflurane anesthesia was purchased from Henry Schein Animal Health (Dublin OH, USA). RNAlater was purchased from Life Technologies (Carlsbad CA). Chelex100 was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Methylene Blue and Eosin were obtained from Ricca Chemical Company (TX USA). Genomic DNA isolation kit was purchased from Promega (Madison Wisconsin, USA). Sheep blood was purchased from Lampire Biological Laboratories (Pipersville PA). PCR primers were purchased from Bio-Synthesis Inc. (Lewisville, TX, USA). Immunocal was purchased from StatLab Medical Products (McKinney TX, USA).

Mice

C57BL6 wild type mice and CB2 receptor deficient mice (CB2^{-/-}), 23 each (males), were purchased from the Jackson laboratory. All experimental procedures were performed in accordance with the Guidelines of the Institutional Animal Care and Use Committee of University of Louisville. The mice received varied in ages by few months. Because of the range of ages, the mice were provided with controls age matched to the knock outs. After matching the ages, the mice were distributed in groups according to the treatment they received.

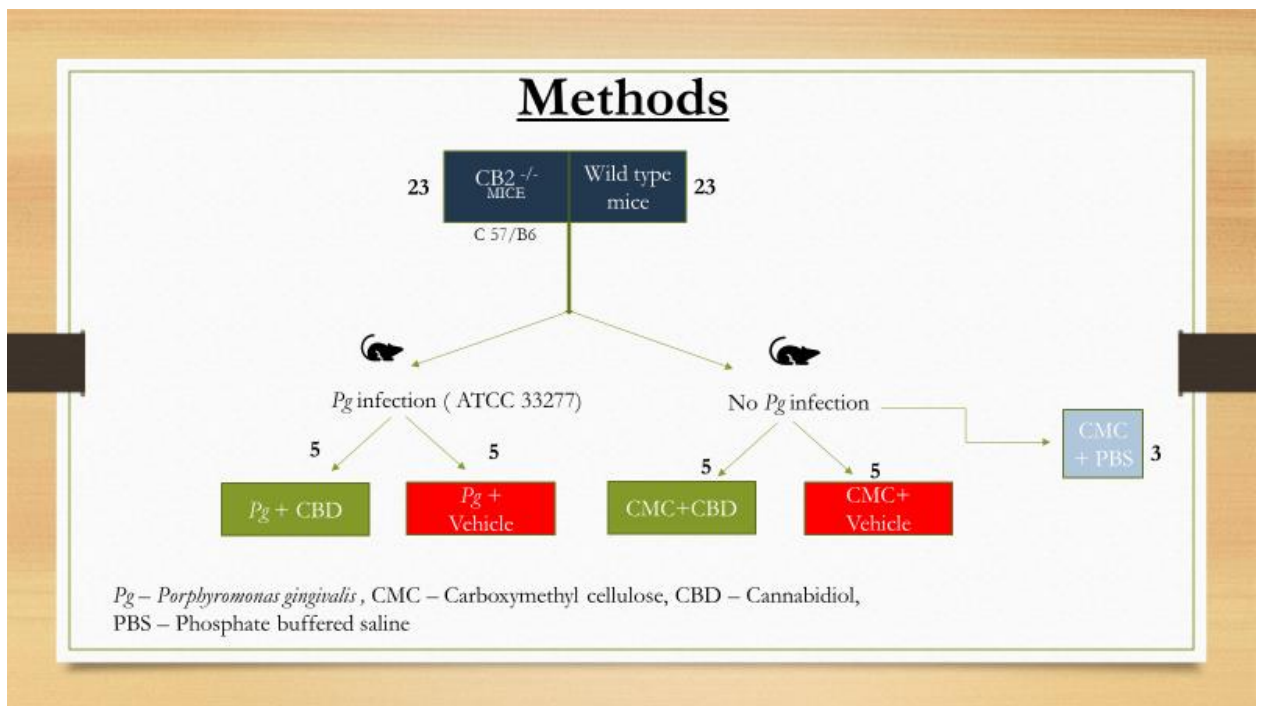


Figure 1 – Mice groups assignment

Both wild type mice and knockout mice were infected with either *P. gingivalis* in 2% CMC vehicle or CMC alone (sham infection) orally. The mice that were infected with *P. gingivalis* were either treated with intraperitoneal injection of cannabidiol or administered vehicle alone. Similarly, the mice not infected with *P. gingivalis* were either treated with intraperitoneal injection of cannabidiol or they were just administered vehicle. Three mice from both, wild type and knock out mice groups were administered carboxymethyl cellulose and phosphate buffered saline.

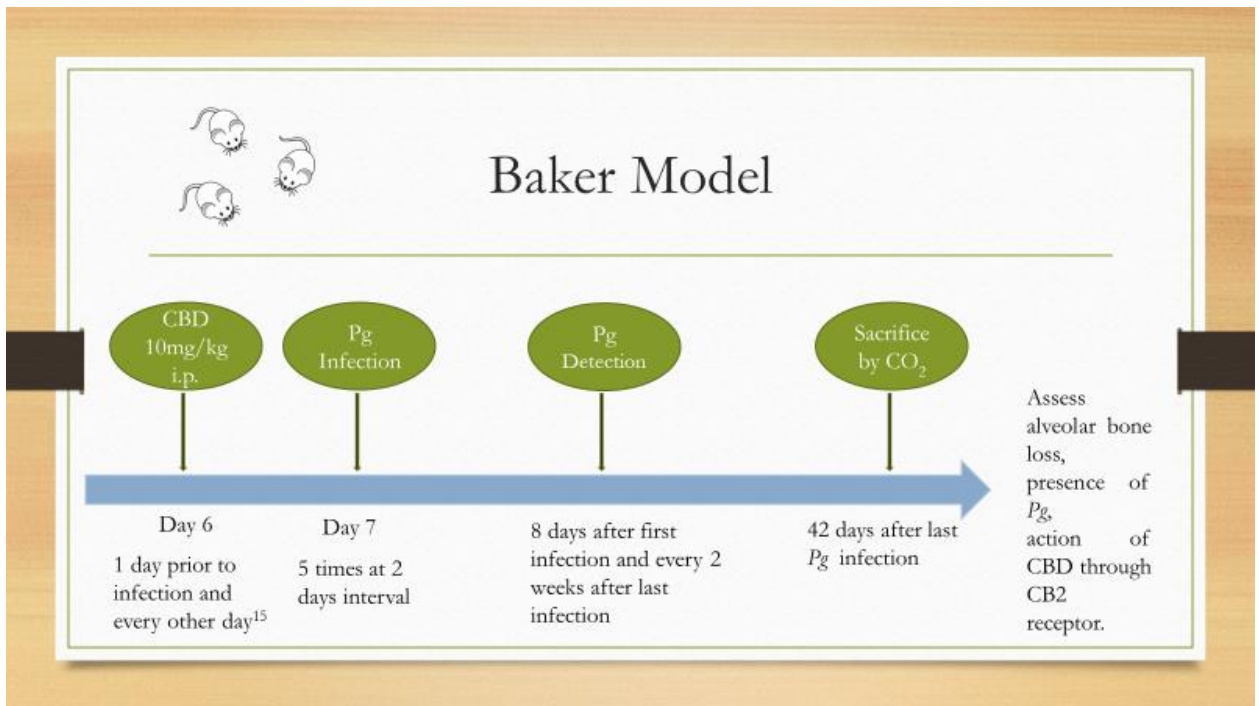


Figure 2 Baker Model

In-vivo mice treatment (Baker Model)

Baker model was followed to infect the mice with *P. gingivalis* and treat them with cannabidiol.

Day 1 – Upon arrival of mice, mice tails were marked from 1 to 23 in both wildtype and CB2 knockout mice.

Day 2 through Day 5 – Mice acclimated to the surrounding environment. No infection or treatment was done at this time.

Day 6 – Mice were treated with intraperitoneal injection of cannabidiol 10 mg/kg with methanol as vehicle. Control mice were administered 10 mg/Kg methanol intraperitoneally. The mice were administered either cannabidiol or methanol every alternate day till the day of sacrifice.

Day 7 – Mice were infected (oral gavage) with *P. gingivalis* (*P. gingivalis* in PBS and carrier CMC) at a concentration of 10^9 cfu/mouse in 100 ul PBS and 2% CMC. Gavage needle was used to infect the mice locally around maxillary molars. To increase the likelihood of infection, mice were anaesthetized with 3% isoflurane and then infected with *P. gingivalis*.

Sham infected mice received 2% CMC in 100µl PBS.

The mice were infected with *P. gingivalis* for a total of five times during the whole process. The infection was done after every two days. The sham infected mice were treated the same way except that they received just PBS and CMC through oral gavage.

P. gingivalis Detection - Oral swabs from all the mice were obtained by swabbing the maxillary gingiva using on-absorbent mini polyester tipped applicator with flexible aluminum handle. This was done to detect the presence of *P. gingivalis* in the oral cavity to assess the effectiveness *P. gingivalis* infections and to monitor the prevalence of *P. gingivalis* in the different treatment groups.

Oral swabs were collected for *P. gingivalis* detection eight days after first infection and then every two weeks after last infection.

""*P. gingivalis* detection was performed by two methods:

""1. Plate culture. The periodontal microbiota was sampled by swabbing around the teeth of maxilla; ""then some of the swabs were submerged into 300ul anaerobic balanced GAM broth. Bacteria were ""extracted by vortexing for 2 minutes. Serial dilutions (10^{-2} , 10^{-3} , 10^{-4}) of the bacterial suspensions ""were plated onto blood agar plates (100 μ l/plate) and CFU were enumerated following anaerobic ""growth for 1 month and examined at 1, 2 and 4 weeks. *P. gingivalis* was identified by black ""pigmented colonies and gram staining.

""2. Standard Polymerase Chain Reaction - Swabs were stored in RNAlater at 4°C. Total DNA was ""extracted from the swabs by using Wizard® Genomic DNA Purification kit (Promega, Cat: ""A1120). Standard PCR procedure and 2% agarose gel electrophoresis was used to detect the *P.* ""*gingivalis* DNA.

""Day 58 - Mice were euthanized 42 days after the last *P. gingivalis* infection. Inhalation of carbon ""dioxide was used as the standard method to sacrifice the mice. The flow of Carbon dioxide was to ""1.5 liters per minute. Carbon dioxide flow was maintained for at least 1 minute after respiratory ""arrest. Decease was confirmed by performing cervical dislocation.

Collection of specimen and in -vitro experiments

1. Serum

Blood was collected from each mouse by cardiac puncture. Sera were stored at -80°C for later assessment. Sera was tested for *P. gingivalis* exposure by analyzing *P. gingivalis* specific IgG and IgM antibodies and total IgG and IgM antibodies, as determined by ELISA.

2. Tissue harvest

Gingiva of the entire maxilla were excised and was immersed in buffer RLT (Qiagen). It was stored at -80°C until used for RNA isolation following the manufacturer's protocol (RNeasy Kit, Qiagen, Valencia, CA).

Real-time quantitative PCR (qPCR) analysis of gingivae was performed to determine cytokine mRNA expression of inflammatory markers (CD 45, IL-1B, mip-2, MMP-8, MMP-9 and CD 14).

Alveolar bone loss evaluation

Freshly harvested skulls were submerged in deionized water and boiled. Following defleshing, the skulls were immersed overnight in 3% hydrogen peroxide. The skulls were immersed for 1 min in 1% bleach then washed and air dried. The maxillae were stained with 0.5% eosin for 5 min followed by 1% methylene blue for 1 minute (both from Ricca Chemical Company, TX, USA).

Alveolar bone loss was measured in millimeters at 14 predetermined points on the maxillary molars of de-fleshed maxillae as the distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC). Bone loss was visualized by methylene blue/eosin staining and quantified using a Nikon SMZ 800 dissecting microscope (40X, Nikon Instruments Inc., Melville,

NY, USA) fitted with a Boeckeler VIA-170K video image marker measurement system (Boeckeler Instruments Inc, Tucson, AZ, USA).

Three-dimensional micro computed tomographic imaging

Three-dimensional state of alveolar bone and volumetric alveolar changes were analyzed by using micro-computed tomography imaging technique.

(A) Scanning

Skyscan micro CT scanner – Skyscan1174v2 and control software Skyscan 114 was used to obtain three dimensional images of mice maxillae.

The following parameters were used for this purpose –

1. Camera = SHT MR285MC
2. Image Pixel Size (um) = 9.70
3. Exposure (ms) = 6000
4. Rotation step (deg) = 0.500
5. Rotation used = 180 degrees
6. Frame Averaging = 3 images were obtained at each rotation
7. Filter = 0.5 um

(B) Reconstruction

Scanned images were reconstructed to stack the images together to produce axial, sagittal and coronal views. Skyscan NRecon software was used for reconstruction of the images obtained by Skyscan scanner (native skyscan data). The native skyscan data is automatically detected by NRecon software. The following settings were used –

1. Postalignment Applied = 1
2. Smoothing = 2
3. Smoothing Kernel = 2
4. Ring Artifact Correction = 10
5. Beam Hardening Correction = 10%

(C) Analysis

Quantitative analysis of the image data set was done for morphometric bone analysis and bone density analysis. Bone volumes of the maxillae were measured by choosing furcation area of maxillary first molar as volume of interest for all the mice maxillae. In the micro Ct software, twenty slices of each reconstructed maxillae image were scrolled through to obtain a complex polygonal 3D volume of interest. This was then used to measure the bone volume.

Calcium hydroxyapatite, phantom materials of known density were used for calibration to determine the bone mineral density. The same complex polygonal 3D volume of interest (furcation area of maxillary first molar) was used to determine the bone mineral density of mice maxillae.

Statistical analysis:

Data were evaluated by ANOVA (InStat v3.06 program, GraphPad) and differences were considered significant at the $p < 0.05$ level.

Human Subjects: No human subjects were used in the experiment.

CHAPTER 3: RESULTS

1. Increased *P. gingivalis*-specific IgG response was seen in *P. gingivalis*-infected CB2 knock-out mice but not wild type mice.

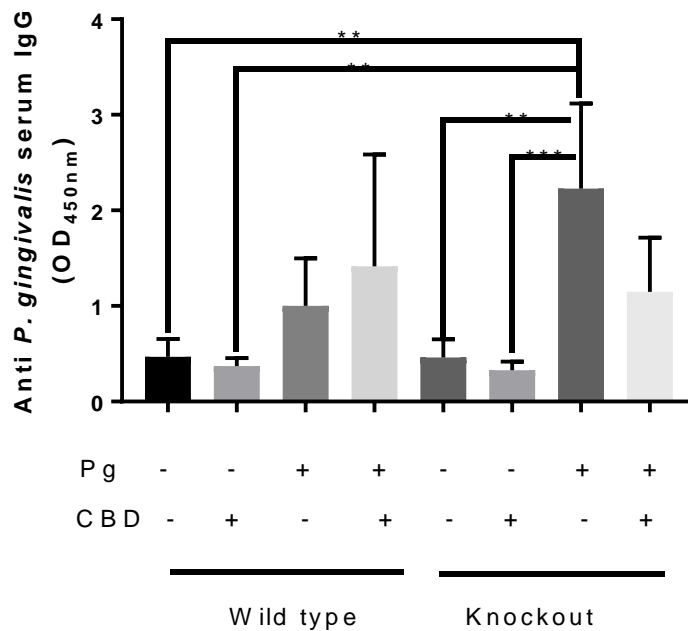


Figure 3.1 - Increased *P. gingivalis*-specific IgG response in *P. gingivalis*-infected CB2^{-/-} but not Wild type mice.

The *P. gingivalis*-specific IgG response was monitored in CB2^{-/-} and Wild Type mice infected, or not, with *P. gingivalis*, in a Baker model of periodontitis at sacrifice. IgG titers were measured in sera by ELISA using *P. gingivalis* (2×10^7 number of *Pg* cells per well) and HRP-labeled horse anti-mouse IgG (1:5,000).

Differences in IgG response between mouse groups (n = 5 per group) were determined by ANOVA with post hoc Tukey-Kramer testing (InStat version 3.10; GraphPad Inc, San Diego California USA). **/** indicate p < 0.01 and 0.001, respectively.

2. Cannabidiol did not affect the levels of serum IgM specific to *P. gingivalis*

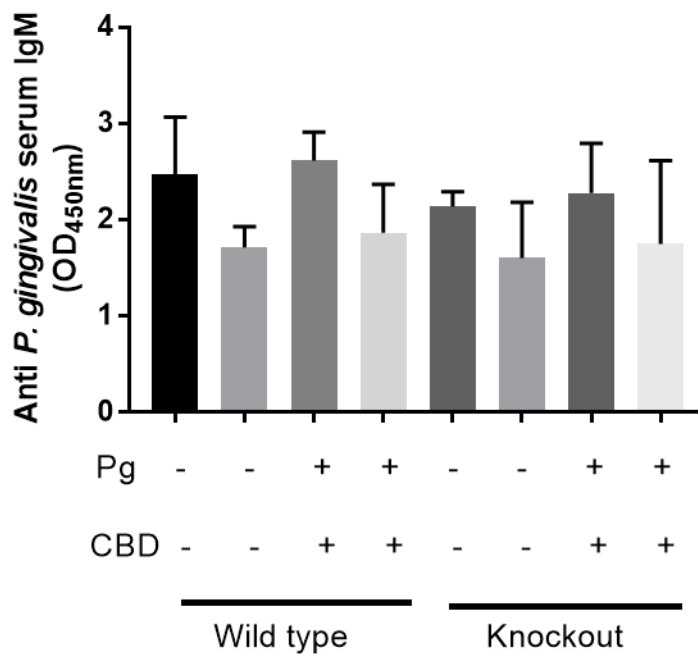


Figure 3.2 - No significant difference was found in the levels of serum IgM specific to *P. gingivalis*

The *P. gingivalis* specific IgM response was monitored in CB2^{-/-} and Wild Type mice infected, or not, with *P. gingivalis*, in a Baker model of periodontitis at sacrifice. IgM titers were measured in sera by ELISA using *P. gingivalis* (2×10⁷ number of *Pg* cells per well) and HRP-labeled horse anti-mouse IgG (1:5,000).

Differences in IgM response between mouse groups (n = 5 per group) were determined by ANOVA (InStat version 3.10; GraphPad Inc, San Diego California USA).

3. Cannabidiol did not affect the levels of serum total IgG.

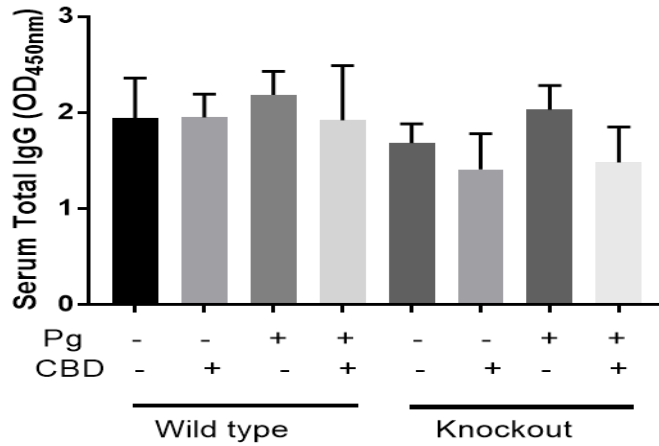


Figure 3.3 – No significant difference found in levels of serum total IgG.

Total IgG response was monitored in CB2^{-/-} and Wild Type mice infected, or not, with *P. gingivalis*, in a Baker model of periodontitis at sacrifice. IgG titers were measured in sera by ELISA using HRP-labeled horse anti-mouse IgG (1:5,000).

Differences in IgG response between mouse groups (n = 5 per group) were determined by ANOVA (InStat version 3.10; GraphPad Inc, San Diego California USA).

4. Cannabidiol significantly reduced the production of total IgM antibodies in sera of *P. gingivalis*-infected wild-type mice but not in knock-out mice.

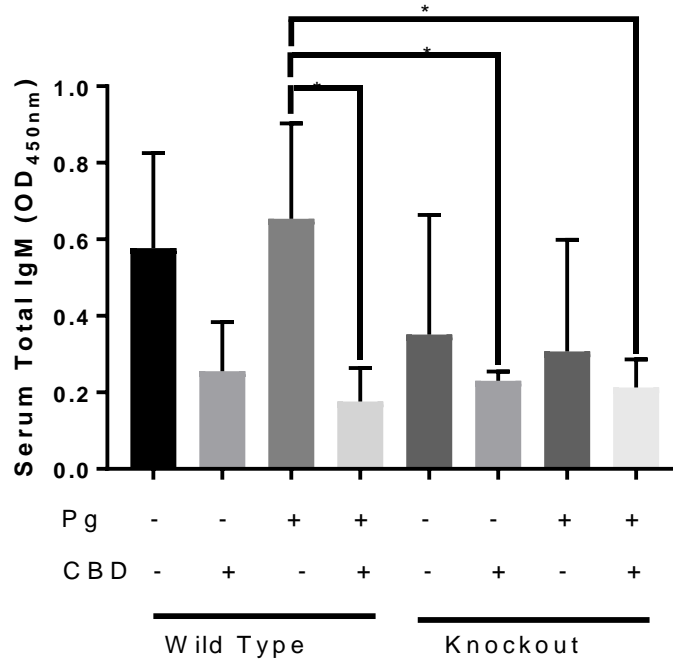


Figure 3.4 – Cannabidiol significantly reduced the production of total IgM antibodies in sera of *P. gingivalis* infected wild-type mice but not in knock out mice.

Total IgM response was monitored in CB2^{-/-} and Wild Type mice infected, or not, with *P. gingivalis*, in a Baker model of periodontitis at sacrifice. IgM titers were measured in sera by ELISA using HRP-labeled horse anti-mouse IgM (1:5,000).

Differences in IgM response between mouse groups (n = 5 per group) were determined by ANOVA with post hoc Tukey-Kramer testing (InStat version 3.10; GraphPad Inc, San Diego California USA). * indicate p < 0.05.

5. Cannabidiol significantly reduced the expression of CD45 in maxillary gingiva in *P. gingivalis*-infected wild-type mice but not in knock-out mice.

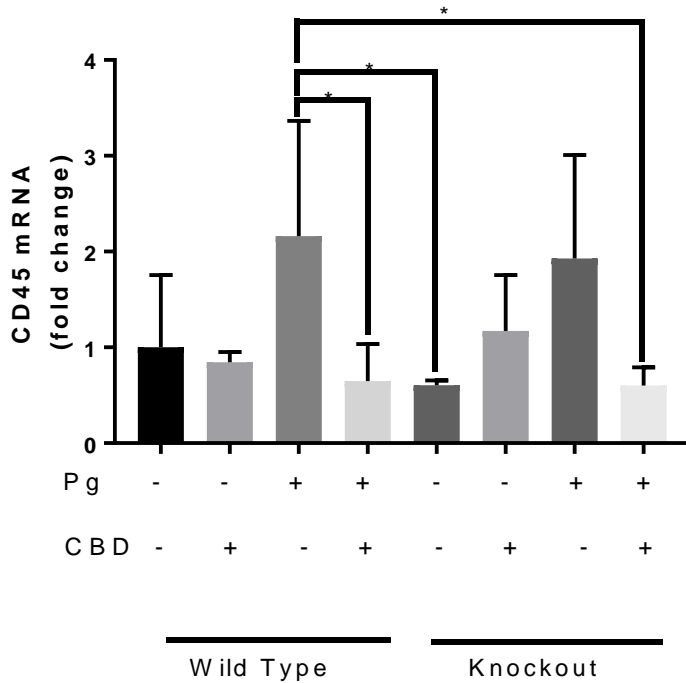


Figure 3.5 - CBD significantly reduced the expression of CD45 in maxillary gingiva in *P. gingivalis*-infected wild-type mice but not CB2^{-/-} mice.

Expression of CD45 mRNA, relative to that of glyceraldehyde 3-phosphate dehydrogenase (housekeeping gene), in the gingivae of mice was assessed using RT-PCR.

Differences in expression of maxillary CD45 mRNA in mice groups (n = 5 per group) were determined by ANOVA with post hoc Tukey-Kramer testing (InStat version 3.10; GraphPad Inc., San Diego California USA).

* indicate $p < 0.05$.

6. Cannabidiol significantly reduced the expression of CD14 in maxillary gingiva of wild type mice infected with *P. gingivalis* but not in knock-out mice.

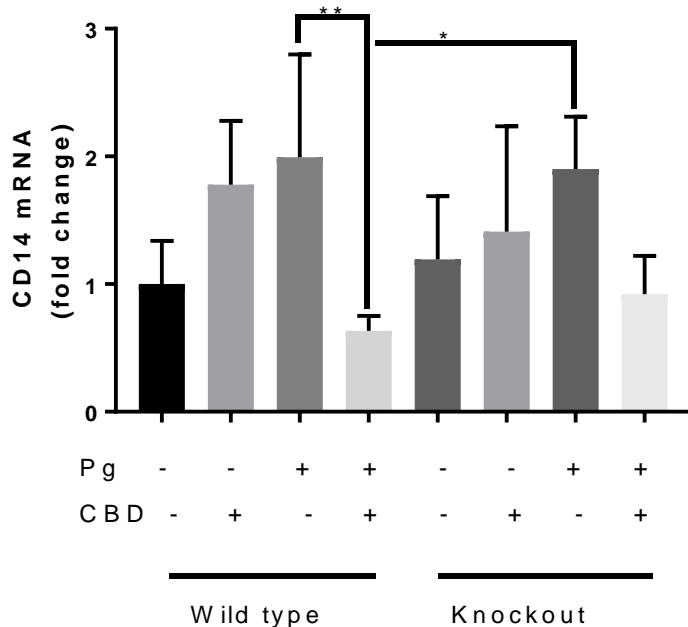


Figure 3.6 – Cannabidiol significantly reduced the expression of CD 14 mRNA in maxillary gingiva of wild type mice infected with *P. gingivalis* but not in knock out mice.

Expression of CD14 mRNA, relative to that of glyceraldehyde 3-phosphate dehydrogenase (housekeeping gene), in the gingivae of mice was assessed using RT-PCR.

Differences in expression of maxillary CD14 mRNA in mice groups (n = 5 per group) were determined by ANOVA with post hoc Tukey-Kramer testing (InStat version 3.10; GraphPad Inc., San Diego California USA).

*/** indicate $p < 0.05/0.01$, respectively.

7. Cannabidiol significantly reduced the expression of IL 1 β in maxillary gingiva of wild-type mice treated with *P. gingivalis* but not in knock-out mice.

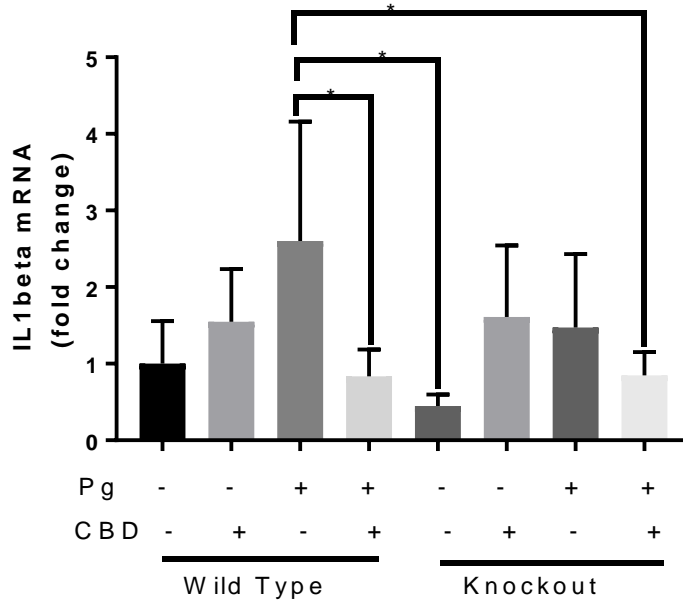


Figure 3.7 - Cannabidiol significantly reduced the expression of IL-1 β in maxillary gingiva of wild-type mice treated with *P. gingivalis* but not in knock out mice.

Expression of IL-1beta mRNA, relative to that of glyceraldehyde 3-phosphate dehydrogenase (housekeeping gene), in the gingivae of mice was assessed using RT-PCR.

Differences in expression of maxillary IL-1beta mRNA in mice groups (n = 5 per group) were determined by ANOVA with post hoc Tukey-Kramer testing (InStat version 3.10; GraphPad Inc., San Diego California USA).

* indicate $p < 0.05$

8. Cannabidiol significantly reduced the expression of MMP-9 in maxillary gingiva of wild type mice infected with *P. gingivalis* but not in knock-out mice.

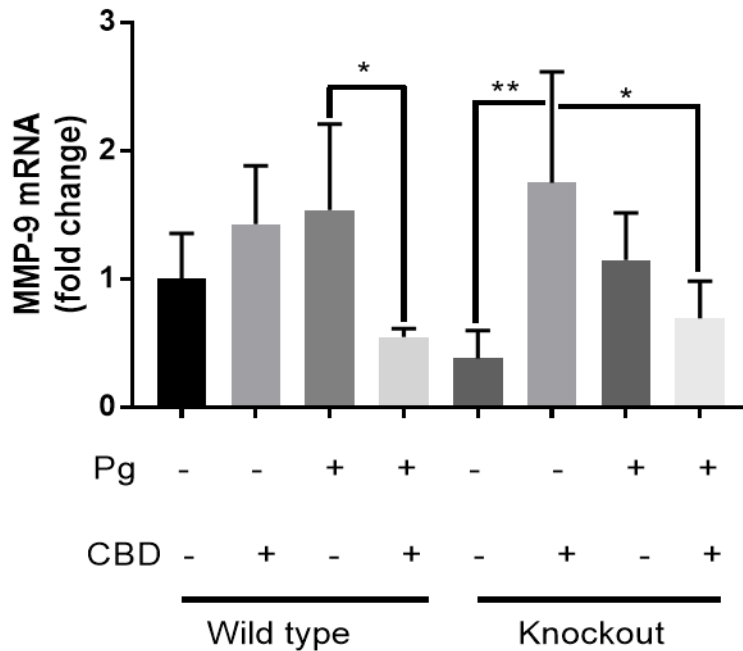


Figure 3.8 – Cannabidiol significantly reduced the expression of MMP-9 in maxillary gingiva of wild type mice infected with *P. gingivalis* but not in knock out mice.

Expression of MMP-9 mRNA, relative to that of glyceraldehyde 3-phosphate dehydrogenase (housekeeping gene), in the gingivae of mice was assessed using RT-PCR.

Differences in expression of maxillary MMP-9 mRNA in mice groups (n = 5 per group) were determined by ANOVA with post hoc Tukey-Kramer testing (InStat version 3.10; GraphPad Inc., San Diego California USA).

*/** indicate $p < 0.05/ 0.01$, respectively.

9. Cannabidiol did not affect the levels of MIP 2 expression between different treatment groups.

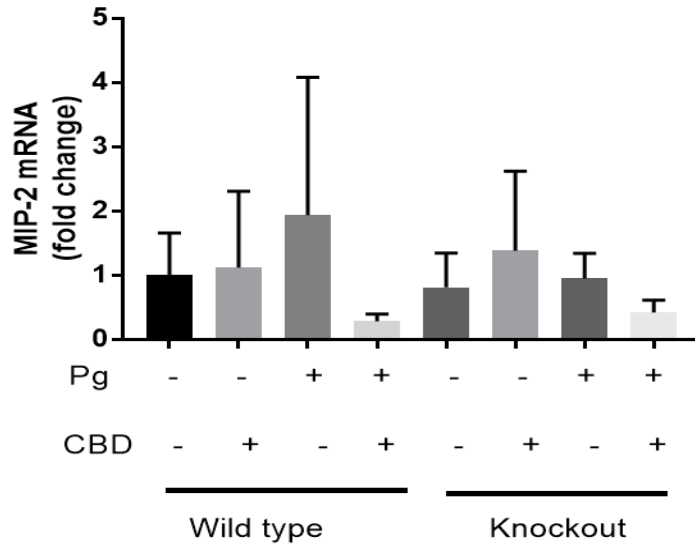


Figure 3.9 – Cannabidiol did not affect the expression of MIP-2 in different treatment groups.

Expression of MIP-2 mRNA, relative to that of glyceraldehyde 3-phosphate dehydrogenase (housekeeping gene), in the gingivae of mice was assessed using RT-PCR.

Differences in expression of maxillary MIP-2 mRNA in mice groups (n = 5 per group) were determined by ANOVA (InStat version 3.10; GraphPad Inc., San Diego California USA).

10. Cannabidiol did not affect the linear alveolar bone levels among different groups.

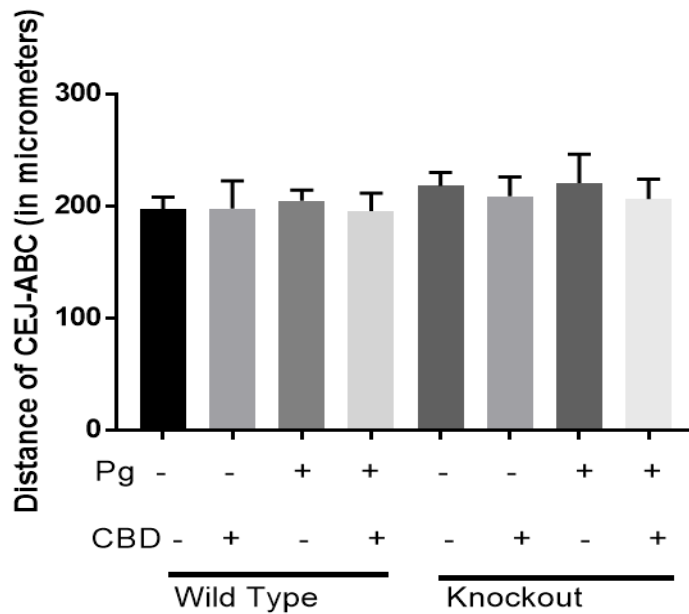


Figure 3.10 – No significant difference was found in linear alveolar bone levels among different groups.

Alveolar bone loss was measured in $CB2^{-/-}$ and Wild type mice, infected or not infected with *P. gingivalis*, in a baker model of Periodontitis at sacrifice. The distance between Cervical-enamel junction and Alveolar bone crest was measured at 14 predetermined sites in maxilla. Measurements were taken by using micro CT.

Differences in bone loss levels between mouse groups ($n = 5$ per group) were determined by ANOVA (InStat version 3.10; GraphPad Inc, San Diego California USA).

11. Cannabidiol did not affect the bone mineral density values among different groups, analyzed by micro CT.

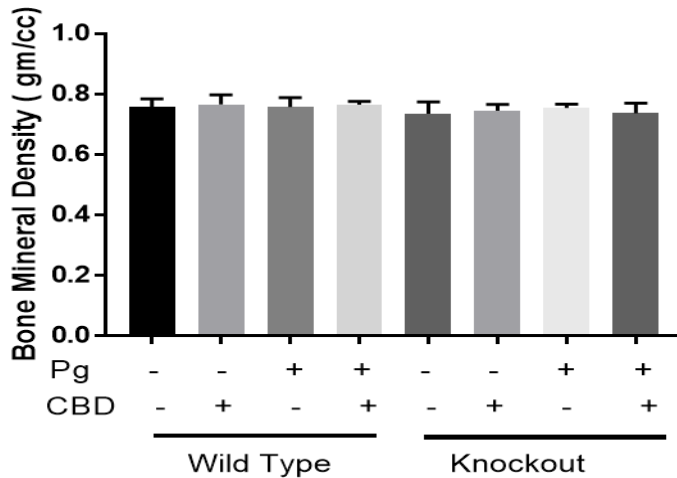


Figure 3.11 - No significant difference was found in the bone mineral density values among different groups.

Bone Mineral Density was measured in $CB2^{-/-}$ and Wild type mice, infected or not infected with *P. gingivalis*, in a baker model of Periodontitis at sacrifice. Mice heads were scanned using micro CT. The maxillary alveolar bone scanned images were reconstructed three dimensionally for assessment of bone mineral density. Maxillary first molar furcation area was chosen as region of interest for all the groups.

Differences in bone mineral density between mouse groups (n = 5 per group) were determined by ANOVA (InStat version 3.10; GraphPad Inc, San Diego California USA).

12. Cannabidiol did not affect the alveolar bone volume among different groups, analyzed by micro CT.

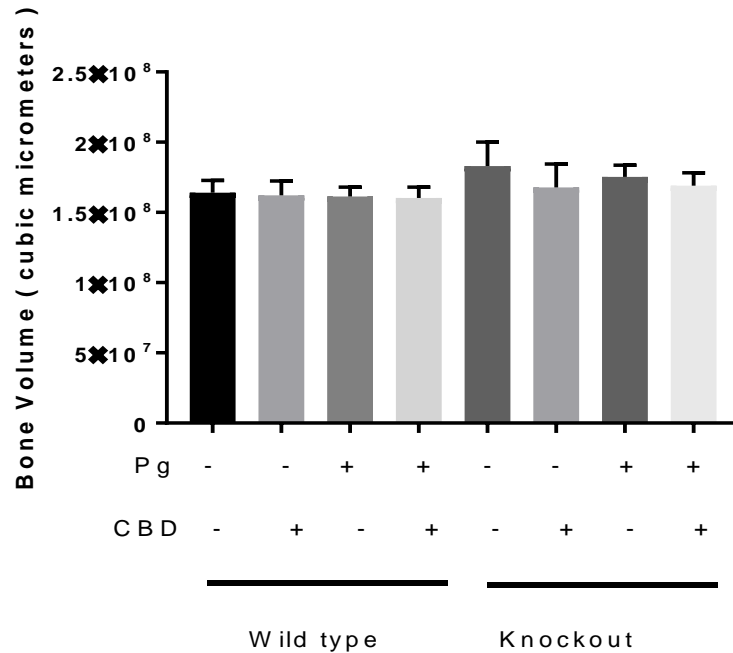


Figure 3. 12 – Cannabidiol did not affect alveolar bone volume among different groups, analyzed by micro CT.

Alveolar Bone Volume was measured in *CB2^{-/-}* and wild type mice, infected or not infected with *P. gingivalis*, in a baker model of Periodontitis at sacrifice. Mice heads were scanned using micro CT. The maxillary alveolar bone scanned images were reconstructed three dimensionally for assessment of bone mineral density. Maxillary first molar furcation area was chosen as region of interest for all the groups.

Differences in alveolar bone volume between mouse groups (n = 5 per group) were determined by ANOVA (InStat version 3.10; GraphPad Inc, San Diego California USA).

Discussion

Periodontal disease is a highly prevalent, multifactorial, chronic inflammatory disease of periodontium eventually leading to destruction of supportive tissues of teeth and tooth loss. The interaction between microbes present in dental plaque and host immune response is a major determinant of progression and clinical manifestations of periodontal disease (Kaur, Grover, Bhaskar, Kaur, & Jain, 2018). *P. gingivalis*, a Gram-negative, black pigmented, assacharolytic and strict anaerobic bacteria, has long been considered an important member of the periodontopathic microbiota involved in periodontal disease progression and bone and tissue destruction (Holt, Kesavalu, Walker, & Genco, 1999). Apart from the periodontopathic microbiota, factors that suppress the immune system have been closely associated with chronic periodontitis. Cannabis is one such emerging risk factor that has been shown to contribute to chronic periodontitis as reported by human observational studies. Cannabidiol, one of the active components of cannabis is closely associated with immune cells and has been shown to exert immunosuppressive effects. Ineffective host immune response may lead to persistence of *P. gingivalis* in the host cells leading to increased alveolar bone loss through direct virulence factors of *P. gingivalis*.

Experimental animal models have been used to understand the various mechanisms behind alveolar bone loss. Mice are the primary *in-vivo* model for human immune system function in health and disease (Mestas & Hughes, 2004).

Three-dimensional state of alveolar bone in the mouse model of periodontal infection has been assessed using micro computed tomography. Micro CT enables detection of intrabody defects and tissues all around the tooth. Due to the high accuracy and sensitivity resulting from the 3-dimensional analysis of the micro-CT technique, fewer animals can be used to obtain significant results (Wilensky, Gabet, Yumoto, Hourri-Haddad, & Shapira, 2005).

In our study we selected furcation area of maxillary first molar as region of interest and determined bone volume and bone mineral density for each scanned and reconstructed maxillary bone. Twenty slices of each bone section were used to determine the values. Cannabidiol did not affect alveolar bone loss, alveolar bone volume and alveolar bone density.

To monitor if cannabidiol alters the immune response in mice infected with *P. gingivalis*, we analyzed the production of antibodies in serum of mice in response to *P. gingivalis* and expression of inflammatory markers in maxillary gingiva of *P. gingivalis* infected mice in presence of cannabidiol. *P. gingivalis* induces strong antibody response (Kinane, Mooney, & Ebersole, 1999) and elevated levels of circulating antibodies *P. gingivalis* in patients with periodontitis have been reported (Chung et al., 2003), (Graswinckel, van der Velden, van Winkelhoff, Hoek, & Loos, 2004). In large population of adults, who participated in the third US National Health and Nutrition Examination Survey, high titers of antibodies against *P. gingivalis* were consistently associated with periodontitis (Dye et al., 2009). In the present study, we examined association between the levels of *P. gingivalis* specific serum IgG and IgM; total IgG and IgM; and the mice infected or not infected with *P. gingivalis*. Cannabidiol significantly reduced the expression of total IgM antibodies in wild type infected with *P. gingivalis*, however

this response was not observed in knock-out mouse. CB2^{-/-} mice infected with *P. gingivalis* showed higher levels of *P. gingivalis* specific serum IgG levels but not wild type mice. If this phenomenon were to happen in humans, it would mean that the adaptive immune response is not well equipped to respond to the challenge in presence of pathogen.

We analyzed effect of cannabidiol on expression of inflammatory markers – CD45, CD14, MMP-9 and IL1 β in the maxillary gingivae of mice. Inflammatory markers mediate the initial immune responses and are crucial for clearance of pathogen and hence resolution of inflammatory disease like chronic periodontitis.

Mouse CD45 (leukocyte common antigen) causes increase in cytokine production and proliferation of T cells (Hermiston et al., 2003). CD45 isoforms are present on all nucleated hematopoietic cells. Since, it appears to play a significant role in the ability of immune cells to respond to activating stimuli (Donovan & Koretzky, 1993), we wanted to determine the effect of cannabidiol on expression of CD45 in maxillary gingivae of mice. In our experiment, cannabidiol significantly reduced the expression of CD45 in *P. gingivalis* infected wild type mice, however this effect was not observed in knock-out mice. Reduced expression of CD45 would lead to insufficient cell mediated immunity to pathogen and hence its persistence.

CD14 is expressed mainly by macrophages and neutrophils. It acts as a co receptor (along with TLR -4) for detection of bacterial polysaccharide (Kitchens, 2000). CD14 is expressed mainly by macrophages and (at 10-times lesser extent) by neutrophils. It is also expressed by dendritic cells (Funda et al., 2001). CD14 plays a pivotal role in TLR4 activation that in turn has a crucial role

in activation of innate immunity and pathogen recognition (Kirschning & Bauer, 2001). In our experiment, cannabidiol significantly reduced the expression of CD14 in wild type mice infected with *P. gingivalis*. This phenomenon was not seen in knock-out mice. Reduced expression of CD14 in response to pathogen would mean increased bacterial invasion and more severe tissue injury.

Matrix Metalloproteinases (MMPs) are zinc-dependent endopeptidases that can cleave extracellular matrix (Basi et al., 2011). MMP-9 levels are increased during inflammatory conditions like periodontal diseases. Degradation of the extracellular matrix and basement membrane leads to the development of periodontitis (Desarda H, Gaikwad, 2012) (Yu & Stamenkovic, 2000). High levels of MMP-9 was found in Gingival crevicular fluid in patients with the periodontitis (Rai, Kharb, Jain, & Anand, 2008). It is mainly released from osteoclasts and polymorphonuclear neutrophils in the periodontal tissues (Ertugrul, Dursun, Dundar, Avunduk, & Hakki, 2013). Levels of MMP -9 levels were found higher in smokers who also had periodontitis as compared to non-smokers (He, Gao, & Jiang, 2016). In our experiment, cannabidiol significantly reduced the expression of MMP-9 in wild type mice infected with *P. gingivalis*. However, this effect was not seen in knock-out mice.

IL-1 β is a member of the proinflammatory IL-1 cytokine superfamily. IL-1 family cytokines are the considered 'early-response' cytokines that are released in the earliest stage of an immune response and act as a trigger for subsequent cascade of proinflammatory cytokines. IL-1 β stimulates the release of IL-6 and IL-17a cytokines (Krishnan, Sobey, Latz, Mansell, & Drummond, 2014). In our experiment, cannabidiol significantly reduced the expression of IL-1 β

in the maxillary gingivae of wild-type mice infected with *P. gingivalis*, however this effect was not seen in knock-out mice. Downregulation of IL-1 β may lead to ineffective innate and adaptive immune responses and hence persistence of pathogen.

Macrophage inflammatory protein (MIP-2) in mice is a member of the alpha chemokine family which also includes human IL-8 (Wolpe et al., 1989). MIP-2 is expressed in epithelial cells after stimulation with Lipopolysaccharide or proinflammatory cytokines (Ohtsuka, Lee, Stamm, & Sanderson, 2001). As with IL-8 in humans, MIP-2 is chemotactic for neutrophils and induces localized neutrophil infiltration, thus regulating the inflammatory cells (Wolpe et al., 1988). In our experiment, cannabidiol did not affect the expression of MIP-2 in the groups of mice infected with *P. gingivalis*.

Cannabidiol significantly reduced the expression of IL-1 β , CD45 and CD14 inflammatory markers in maxillary gingiva in *P. gingivalis* infected wild type mice but not CB2 $-/-$ mice. Deficient immune response to *P. gingivalis* may lead to its persistence and more severe periodontal destruction in cannabis users. This is analogous with in-vitro nicotine studies and with clinical studies showing more severe clinical manifestations of chronic periodontitis in tobacco smokers. However, our results showed a reduction in MMP-9 expression in presence of cannabidiol. This anomaly is hard to explain and does not correspond with the findings in cigarette smokers where MMP-9 levels are significantly higher.

Increased bone loss was hypothesized because we know cannabis drives bone loss in animals and humans. This did not happen here. May be because we didn't have persistent infection. However, immune differences between wild type versus knock out mice that we did see could have a long-term effect in chronic infection.

In summary, cannabidiol alters the immune response to *P. gingivalis* in mice by affecting the secretion of immunoglobulins and expression of inflammatory markers. Suppression of immune response by cannabidiol can lead to increased periodontal destruction in presence of *P. gingivalis*. Although no difference in bone loss was found in this model, the effect on inflammatory mediators by cannabidiol was significant. This could have an impact on chronic periodontitis in due course of time in presence of persistent pathogenic infection.

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CURRICULUM VITAE

Name : Shilpa Singh

ADDRESS : Department of Oral Immunology and Infectious Diseases
University of Louisville School of Dentistry
501 South Preston Street
Louisville KY 40202

EDUCATION AND TRAINING

Bachelor of Dental Surgery
Maharshi Dayanand University, India
2005-2010

PROFESSIONAL SOCIETIES

American Association of Dental Research
American Dental Association

DENTAL EXPERIENCE

Associate Dentist (October 2010 – December 2013)
India