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<https://doi.org/10.18297/etd/2503>

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CANNABINOIDS SUPPRESS THE INNATE IMMUNE RESPONSE TO
PERIODONTAL PATHOGEN *PORPHYROMONAS GINGIVALIS* IN
GINGIVAL EPITHELIAL CELLS

By

Rajarshi Guha Niyogi

A Thesis Submitted to the Faculty of the
School of Dentistry of the University of Louisville in
Partial Fulfillment of the Requirements for the Degree of

Master of Science in Oral Biology

Department of Oral Health and Systemic Disease
School of Dentistry
University of Louisville
Louisville, KY

August 2018

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A Thesis approved on

June 6, 2016

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.....F GF IECVKQP "

"

.....This Thesis is dedicated to my parents,

"

Dr. Sarbajit Guha Niyogi

"

"And

"

" Mrs. Madhumita Guha Niyogi

"

For their invaluable support and continuous encouragement

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr .David A Scott for providing this huge opportunity to work under him. None of this would not have been possible without his endless effort to help me to improve all the way through my journey in an entirely new place.

I would also like to thank my committee members, Dr. Richard Lamont and Dr. Huizi Wang for their constant guidance, support and patience. And Professors Dr. Douglas Darling, Dr. Donald Demuth, Dr. Shuan Liang and Dr. Jan Potempa for being great teachers.

I wish to thank Diane E Renaud my lab Manager for her constant support in this journey. I also want to thank Gweneth Lamont and Zhen Gu for their guidance and teaching me techniques related to my project. And Hima, Neelima and Atul for keeping me going in the lab. I am very thankful to Dr. Justin Hutcherson for his guidance

through my initial months into this project.

Lastly my sincere thanks to my parents Dr. S. G. Niyogi, Mrs. M. G. Niyogi, Shashi and Mahesh Chaturvedi for their relentless support through all my endeavors. I also wish to thank my sibling Roshni for encouraging through difficult times. I also want to convey my sincere thanks to Richa and Ria my wife and daughter without their constant encouragement and support I would not have been able to accomplish my dream.

ABSTRACT

CANNABINOIDS SUPPRESS THE INNATE IMMUNE RESPONSE TO THE PERIODONTAL PATHOGEN *PORPHYROMONAS GINGIVALIS* IN GINGIVAL EPITHELIAL CELLS

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June 6, 2016

Marijuana is widely used in the United States for recreational and medicinal purposes. Despite the proposed beneficial effects of cannabis on certain medical conditions, there is also a concern there is also a concern over negative health consequences of marijuana smoking. Recently, cannabis use has been established as a dose-related risk factor for chronic periodontitis. Although the induction / exacerbation of chronic periodontitis in marijuana users has been established epidemiologically, the mechanisms underlying such predisposition are still unknown. It has long been suggested that marijuana has anti-inflammatory properties, while human epithelial cells are known to express cannabinoid receptor type 2 (CB2). Therefore, we hypothesized that marijuana-derived cannabinoids may suppress the innate immune response, altering the ability of gingival epithelial cells to respond appropriately to bacterial stimuli (the classic TLR4 ligand, *E. coli* LPS; and the major periodontal

pathogen, *Porphyromonas gingivalis*). More specifically, we hypothesized that predominant phytocannabinoid subtypes (cannabidiol [CBD], cannabinol [CBN], or tetrahydrocannabinol [THC]) at physiologically relevant doses (0 - 10 µg/ml) would suppress proinflammatory cytokine production in LPS- (0-1 µg/ml) or *P. gingivalis* (MOI, 10:1) exposed telomerase immortalized human gingival keratinocytes (TIGK cells) in a CB2-related manner. We also hypothesized that marijuana-derived cannabinoids may influence the viability of both epithelial (TIGK cells, as assessed by Trypan blue exclusion) and representative oral bacteria (*P. gingivalis*, *Treponema denticola* and *Filifactor alocis*, growth curves monitored by optical density). Higher doses (>5 µg/ml) of marijuana derived cannabinoids (CBD, CBN or THC) inhibited the growth of *P. gingivalis* ($p < 0.001$) and *F. alocis* ($p < 0.001$), relative to unexposed bacteria, whereas *T. denticola* growth was resistant to all cannabinoid doses tested (1- 10 µg/ml, $p > 0.05$). TIGK cells were non-viable when exposed to high cannabinoid concentrations (> 10 µg/ml). Sub-lethal (1 -5 µg/ml) doses of each cannabinoid subtype suppressed the production of IL-8 and IL-6 but enhanced IL-10 release in *P. gingivalis* or LPS-stimulated TIGKs (all $p < 0.001$). Treatment with the CB2 inhibitor, JTE907, did not rescue cannabinoid-induced immune suppression, suggesting that alternative cannabinoid receptors, such as CB1, GPR55 or A2A receptors, may be associated with the anti-

inflammatory function of marijuana-derived cannabinoids. If the phenomena of (i) cannabinoid induced epithelial toxicity; (ii) growth restriction of a sub-population of bacterial species in the oral biofilm; and (iii) innate suppression in gingival epithelial cells, established herein, occur *in vivo*, they are likely to help explain increased susceptibility to periodontal diseases in cannabis users. To expand, the epithelial barrier may be compromised in marijuana smokers, facilitating bacterial entry and more efficient diffusion of microbial toxins into the gingival tissues; marijuana-derived cannabinoids may promote microbial dysbiosis; and a suppressed pro-inflammatory epithelial response to bacterial stimuli may promote immune evasion, colonization and persistence by pathogenic bacteria. These data provide some of the first mechanistic insights into cannabinoid associated periodontal diseases. Such mechanistic advance are critical if novel therapeutics to prevent chronic periodontitis in marijuana users are to be developed. Further, an improved understanding of the immunoregulatory properties of cannabinoids will be useful in the development of therapeutics for the containment of inflammation, in general

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CHRONIC PERIODONTITIS

Chronic periodontitis is an inflammatory condition affecting a large section of the global adult population. It is a disease which affects the supporting structures of the teeth causing marked degeneration and destruction of these structures, potentially leading to the exfoliation of the teeth. The host immune response plays a critical role in the progression of chronic periodontitis. About 47.2% of the population is affected by the disease and consumes \$14 billion as health cost in United States^{1, 2}. Periodontitis is usually seen affecting the adult population more although an acute aggressive variant is also noted in young adults which is characterized by the extensive bone loss around the lower incisors and first molars. Chronic periodontitis is multifactorial disease and its progression depends on the interplay of both intrinsic factors, like the immune response, and extrinsic environmental factors, like smoking and oral hygiene habits³.

In humans, the epithelial layer of mucosal surface represents an initial interface for colonizing microorganisms⁴. In addition to constituting a

physical barrier to the entry of organisms, epithelial cells can sense and respond to the presence of bacteria through an elaborate signaling network that can communicate information to the underlying immune effector cells^{5, 6}. Environmental factors such as smoking can expose the epithelial mucosa to numerous compounds which may compromise these essential signaling pathways leading to a suppressed innate response which predisposes to periodontal pathogen infection, suppresses pathogen clearance, and increases susceptibility to chronic periodontitis

IMMUNITY AND INFLAMMATION

Immunity is defined as the ability of the body to resist or fight off infection and disease. Innate immunity, also known as the nonspecific immune system, is the first line of defense⁸. It is a part of the overall immune system that comprises the cells and mechanisms that defend the host from infection by other organisms. This means that the cells of the innate system recognize and respond to pathogens but do not confer long-lasting or protective immunity to the host. Innate immune systems provide immediate defense against infection⁶. The major functions of the innate immune system include:

- Recruiting immune cells to sites of infection, through the production of chemical factors, including specialized chemical mediators called cytokines.
- Activation of the complement cascade to identify bacteria, activate cells, and promote clearance of antibody complexes or dead cells.

- The identification and removal of foreign substances present in organs, tissues, the blood and lymph, by specialized white blood cells.
- Activation of the adaptive immune system through a process known as antigen presentation.
- Acting as a physical and chemical barrier to infectious agents.

Adaptive immunity on the other hand refers to antigen-specific immune response. The adaptive immune response is more complex than the innate. The antigen first must be processed and recognized. Once an antigen has been recognized, the adaptive immune system creates an army of immune cells specifically designed to attack that antigen⁹.

Inflammation is part of the complex biological response of vascular tissues to harmful stimuli such as pathogens, damaged cells and other irritants. It is a protective response involving host cells, blood vessels, proteins and other mediators that are intended to eliminate the initial cause of cell injury and insult¹⁰. The classical signs of inflammation are pain, heat, redness, swelling, and loss of function. Inflammation is a stereotypical response, and therefore it is considered as a mechanism of innate immunity as compared to adaptive immunity¹¹, which is specific for each path

THE PERIODONTAL PATHOGEN - *P. GINGIVALIS*

The periodontal pathogen, *P. gingivalis* is an anaerobic, Gram negative bacterium that is considered to be a major etiological factor in chronic periodontitis¹². It is a non-spore forming and belongs to the phylum *Bacteroidetes*. This opportunistic pathogen is known to cause disease by promoting shifts in the oral flora that lead to inflammation and bone loss¹³,¹⁴. *P. gingivalis* grows *in vitro* by forming smooth, raised colonies on blood agar plates. The colonies appear creamy to white in color initially and turn to black in three to four days with a specific unpleasant odor. Bacterial colonies turn black due to the uptake of hemin from blood in the plates¹⁵. Morphologically, the bacterium appears cocco-bacillar (small rods) and is asacchrolytic and thus metabolizes the host proteins for its growth and survival.

P. gingivalis have been considered as a “keystone” pathogen in periodontal disease as it has a disproportionately large effect on its

biofilm community when compared to its abundance¹⁶. The presence of low numbers of *P. gingivalis* was required for the significant increase in oral microbiota which was associated with disease (PSD model)¹⁷. The total percentage of abundance of *P. gingivalis* in few cases was found to be less than 0.01% of oral commensal bacteria in humans. The bacterium is also responsible for altering the normal host-bacterial inter play in the periodontium¹⁸. It suppresses the complement system and thereby provides an excellent niche for other commensal bacteria to grow and invade further, leading to periodontitis.

The polysaccharide capsule is a potent virulence factor in *P. gingivalis*. It has mechanisms that down-regulate specific aspects of host immune response and thereby survives proficiently by masking the host immune system^{19,20}. The capsule of *P. gingivalis* is thought to make a significant contribution to virulence of the bacterium. The non-encapsulated strains would probably be less efficient in progression of periodontitis due to their reduced ability of both biofilm participation and tissue invasiveness²¹. It is indicated that the capsule plays multiple roles in the virulence of *P. gingivalis*. These include reduction of the host immune response to the bacterium, reduced phagocytosis, and increased bacterial survival^{21,22}.

P. gingivalis has long been considered an important member of the periodontopathic microbiota involved in periodontal disease

progression, bone and tissue destruction. *P. gingivalis*, because of its ability to produce significant numbers of potential virulence molecules, is considered an important pathogen in this progression from health to disease²³

P. gingivalis has two dominant kinds of fimbriae, major and minor. The fimbriae play a critical part in the bacterial virulence. Six variants of major fimbriae are known to exist depending on the genetic diversity of the gene coding them (fim A). Type II fimbriae are known to be associated with higher adhesion and invasive potential in comparison to others. The minor fimbriae (mfa1) aid in cohesion with other bacteria (*Streptococcus gordonii*) and thereby biofilm development and growth²⁴. The lipopolysaccharide (LPS) of *P. gingivalis* is considered to be a potent virulence factor too. The LPS stimulates the host inflammatory pathway by interacting with the Toll-like Receptor 4 (TLR 4)^{25, 26}. Interestingly, the LPS of *P. gingivalis* exists in varying chemical structure, each with different properties. The tetra-acylated form of LPS is a TLR 4 antagonist whereas the penta-acylated form is a TLR 4 agonist. The shift from penta-to tetra-acylated forms is regulated by the presence of hemin (increased hemin levels up regulate tetra-acylation)²⁷.

Gingipains are cysteine proteases that provide maximal advantage to the bacterium in the exacerbation of periodontal disease. More than 85% of the proteolytic activity of the bacterium is accountable to the

existence of gingipains. These are encoded by three genes *rgpA*, *rgpB* and *kgp*. *rgpA* and *rgpB* specifically cleave at arginine peptides and *kgp* at lysine residue²⁸. These play a multi-functional role in the bacterium and are thereby considered vital for the survival of the pathogen. *rgpA* is required for the maturation of long/major fimbriae²⁹. Thus, gingipains indirectly play a role in the attachment of *P. gingivalis* in the biofilm. These proteases also enhance the nonfimbrial adhesion mechanism by binding closely to epithelial cells, fibroblasts and extra cellular matrix proteins including fibrinogen, type V collagen, laminin and fibronectin. The destruction of extra cellular matrix leading to progression of periodontitis is attributed to gingipains³⁰. Additionally, these proteolytic enzymes help the bacterium in nutrient acquisition. Iron uptake by *P. gingivalis* is attributed to the presence of hemagglutinins, which in turn are regulated by gingipains³¹.

Furthermore, the bacterium also obtains peptides as a source of carbon and nitrogen through gingipains. The gingipains, being endonucleases, cleave the host proteins which are then converted to di-tripeptides by the respective peptidases³². Finally, the di-tripeptides are transported into the cell and then metabolized for energy needs. Gingipains also neutralize and subvert the host immune responses by cleaving several products in the pathway of complement activation in the host³³. Pathogenic strains of *P. gingivalis* are active

participants of the complex oral biofilms. Bacterial interactions through quorum sensing, cross feeding, co-aggregation, contact dependent signaling aid in the metabolic exchange, alteration of gene expression and efficient survival of the organism in the biofilms³⁴.

The interplay not only effectively improves the pathogenicity of *P. gingivalis* but also of the entire community. The initial colonization of *P. gingivalis* in the oral cavity occurs by the adhesion to primary colonizers, such as *Streptococcus gordonii*²⁴. The minor fimbrial antigen (mfa1) of *P. gingivalis* adheres to the streptococcal antigens I and II. The interactions also aid in the survival of the bacteria in adverse conditions. The ability of the bacterium to co-aggregate and associate with several other bacteria in the biofilm, in turn up regulates the virulence of the entire community¹⁷. *P. gingivalis* invades several host cell types including gingival epithelial cells, endothelial cells and smooth muscle cells³⁵.

The inter-cellular spreading of *P. gingivalis* is through a unique mechanism. The bacteria spread between the cells through actin based membrane protrusions. This mode of inter-cellular translocation prevents the bacterium from being exposed to the host's immune responses. Studies on the invasion process have suggested the presence of a unique mechanism of invasion into the host. The serine phosphatase enzyme of *P. gingivalis* is involved in this process. The enzyme activity leads to dephosphorylation and activation of cofilin,

a protein responsible for actin remodeling through de
polymerization³⁶. Although the bacterium can spread without cell-to-
cell contact, the tight cell contact hastens the invasion process. *P.*
gingivalis establishes a long term cohabitation with gingival epithelial
cells, which in turn can survive up to eight days after infection with
the bacteria³⁷. Inside the cell, the bacteria migrate to the perinuclear
area and accumulate. The bacterium resides in the host cells,
overcoming the major challenges like oxidative stres

OTHER REPRESENTATIVE PERIODONTAL PATHOGENS

Cv'c"o letqdkn'rgxgn"vj g"qyj gt"dcevgtk"o quv'eqo o qpn' "cuuqekcvgf "y kj "

ej tqple'r gtkqf qp'vku'ctg"Vtgr qpggo c'f gp'keqr "Vf +0Qtcn'ur ktqej gvgu"

qeewr { "c"wpks wg"plej g'lp"vgtu u'qh"gp'xktqpo gp'v'cpf "vj gk'r qn' (o letqdkn'

pcwtg0Vtgr qpggo gu'r quugu"cy kf g'tcpi g'qh'xktwgpeg'hcevtu"vj cv"

r tqo qvg'uwtxkcn'cpf 'r cvj qi gplek' { 'lp"vj g'i lpi kcn'r qengv⁵: 0T gegpv"

gzco r ngu'qh'o qdkp'FP C"grgo gpw."i gpgve"gz ej cpi g'cpf "dcevgtkqr j ci gu"

j ki j rki j v'vj g'eqo r rnzkv' { "qh'lp'vgtcevkpu'dgy ggp"qti cpkuo u'lp"vj g'qtcn'

ecxkv' 0T gegpv'tgugctej "j cu"cnq'hqewugf "qp"j qy "qtcn'Vtgr qpggo gu'ugpug"

cpf 'tgur qpf "v'vj g'f { pco le"gp'xktqpo gpw0Vj g' { "j cxg"o wnr ng"VEUu"cpf "

ej go qvczk/ugpukpi 'tgegr vqtu."cpf "o c' { "tgur qpf "d' { "meqo qv'qp"cpf "

xktwgpeg"gzr tgu'kp0Vj g' { "cnq"gzr tguu'o wnr ng'wr vng"cpf 'tgi wv'vt { "

u' { ugo u'hqt"pwt'kp'v'ces wku'kqp0Qtcn'ur ktqej gvgu'ch'gev'o wnr ng'j quv'egm'

v' { r gu0P qvcdn' . "vj g' { "ecp"ce'v'xcv'g'lo o wpg'tgur qpugu."rgcf lpi "v' "vkuuwg"

lplwt { . "dw'lo r ckt"uqo g'etwekn'lp'pcv'g'tgur qpugu."lpenf lpi 'pgwtqr j kn'

hwpe'v'kp"cpf "VNT"ce'v'xcv'kqp."r t'gxgp'v'kpi "vj gk"qy p'gtcf kec'v'kqp⁵: 0H'kpcn' { . "

qtcn'Vtgr qpggo gu'j cxg"o cp' { "eqpugt'xgf "cu'y gm'cu'uqo g'wpks wg'xktwgpeg"

r tqr gt'v'ku⁶²⁰

The other fast emerging periodontal pathogen is *F. alocis*⁴¹. *F. alocis* is one of a few bacteria that is associated with multiple oral diseases including periodontitis, localized aggressive periodontitis, endodontitis and peri-implantitis. Its relative abundance in the periodontal pocket of patients with periodontitis may support the hypothesis to include *F. alocis* as a diagnostic marker organism⁴². This organism has unique potential virulence characteristics such as resistance to oxidative stress and genes coding for a well-developed amino acid metabolic pathway that can modulate multiple changes to the oral microbial community and the host cell proteome that collectively can lead to the disease process⁴³. Multiple studies have documented the increasing incidence and importance of *F. alocis* (In comparison to the other traditional periodontal pathogens, the high incidence of *F. alocis* in the periodontal pocket compared to its absence in healthy or periodontitis resistant patients has highlighted its importance in the infectious disease process. *F. alocis* has also been discovered in the canals of root-filled teeth with periapical lesions and is associated with signs and symptoms of endodontic infections⁴⁴. It has also been identified as one of the prevalent phylotypes in cases of failed endodontic treatment. *P. gingivalis* ATCC 33277 co-cultured with *F. alocis* showed significant increase in biofilm formation. This enhanced biofilm forming capacity may be due to the ability of both species to auto aggregate and express unique

components. This could indicate a commensal relationship between *F. alocis* and *P. gingivalis*. Thus, *F. alocis* and *P. gingivalis*, each with different growth rates, could form a mixed species biofilm and coexist. As a result, *F. alocis* proteins could enable *P. gingivalis* to proliferate and disseminate from these biofilms thus facilitating its virulence⁴⁵.

CANNABINOIDS:

Cannabis, commonly known as marijuana, is a product of the *Cannabis sativa* plant and the active compounds from this plant are collectively referred to as cannabinoids⁴⁶. Cannabinoids are a group of compounds that mediate their effects through cannabinoid receptors. The discovery of Δ^9 tetrahydrocannabinol (THC) as the major psychoactive principle in marijuana, as well as the identification of cannabinoid receptors and their endogenous ligands, has led to a significant growth in research aimed at understanding the physiological functions of cannabinoids⁴⁷. Cannabinoid receptors include CB1, which is predominantly expressed in the brain and mediates the psychotropic side effects, and CB2, which is primarily found on the cells of the immune system⁴⁸. Other sub-types of cannabinoids include cannabidiol (CBD) and cannabinol (CBN). The fact that both CB1 and CB2 receptors have been found on immune cells suggests that cannabinoids could play an important role in the regulation of the immune system⁴⁹. Cannabinoids are potent anti-inflammatory agents and they exert their effects through suppression of cytokine production, inflammatory cell migration, and induction of T-regulatory cells and induction of apoptosis⁵⁰.

The various mechanisms through which cannabinoids bring about anti-inflammatory action are as follows:

A) CANNABINOIDS MODULATE CYTOKINE PRODUCTION:

Cytokines are the signaling proteins synthesized and secreted by immune cells upon stimulation. They are the modulating factors that balance initiation and resolution of inflammation. One of the possible mechanisms of immune control by cannabinoids during inflammation is the dysregulation of cytokine production by immune cells^{51, 52}. Furthermore, cannabinoids may affect immune responses and host resistance by perturbing the balance between the cytokines produced by T- helper subsets, Th1 and Th2.

Earlier studies have showed that THC have produced a concentration-dependent inhibition of T cell proliferation⁵³. The production of cytokines by stimulated T cells plays a critical role in amplifying and targeting immunity. When activated T cells mature into Th1 cells, they secrete IL-2 and IFN- α and potentiate cell-mediated immunity by enhancing T cell proliferation and effector functions, up-regulating MHC expression, promoting IgG2a isotype switching, and activating effector cells such as NK cells and macrophages. In contrast, when activated T cells acquire a Th2 phenotype, they secrete IL-4, IL-5 and/or IL-10, and

potentiate allergic/atopic antibody responses while suppressing T cell proliferation and cell mediated immunity⁵³.

B) CANNABINOIDS MODULATE INFLAMMATORY-CELL MIGRATION:

The accumulation of leukocytes in tissues contributes to a wide variety of diseases; these 'molecular codes' have provided new targets for inhibiting tissue-specific inflammation. However, immune cell migration is also critically important for the delivery of protective immune responses to tissues. Thus, the challenge for the future will be to identify the trafficking molecules that will most specifically inhibit the key subsets of cells that drive disease processes without affecting the migration and function of leukocytes required for protective immunity. Inflammatory migration of immune cells is involved in many human diseases. Identification of molecular pathways and modulators controlling inflammatory migration could lead to therapeutic strategies for treating human inflammation-associated diseases. Although the exact mechanism is unknown but studies have highlighted several signaling pathways that may suppress the leukocyte inflammatory migration. Cannabinoids have been found to suppress inflammatory cell migration in some studies but exact mechanism still needs to be established. Few possible mechanism through which cannabinoids can suppress inflammatory cell migration through CB2 receptor are as follows:

- One of the studies have shown that CB2 receptor agonist have an inhibitory action on the JNK/c-Jun/Alox5 Pathway⁵⁴. The cooperative effect between the Cnr2 agonist and the Alox5 inhibitor of leukocyte inflammatory migration implicates a new strategy for developing anti-inflammation therapies. Investigating the effect of a combined therapy with Cnr2 agonist and Alox5 inhibitor would be valuable in treating human diseases such as multiple sclerosis, rheumatoid arthritis, atherosclerosis, asthma, or even cancer.
- It has also been seen that cannabinoid receptor 2 agonist reduces immune cell migration in neuro inflammation via inhibition of matrix metalloproteinase-9⁵⁵. The study was done using murine model and dendritic cell to conduct migration assay and see the effect of cannabinoids on the migration of these cells were studied. The Cannabinoid mediated suppression of leukocyte adhesion and migration resulting from an inhibition of TH1 cell cytokine production.

C) CANNABINOIDS REGULATE HELPER T CELLS:

Cannabinoids have a Th-cell biasing effect, in which Th1-cell activity is suppressed and Th2-cell activity is increased⁵⁶.

From prior studies, it seems that cannabinoids bias the immune response away from Th1-cell immunity and that cannabinoid receptors are involved in this process³⁰.

Treatment with cannabinoids has been shown to alter the expression of specific transcription factors, the expression of the Th2-cellpromoting transcription factor GATA-binding protein 3 (GATA3) has been found to be increased by treatment with cannabinoids whereas the production of IL-2 expression-promoting transcription factors is suppressed by treatment with cannabinoids. The selective suppression of Th1-cell immunity by these drugs supports their potential use in the treatment of chronic inflammatory diseases. There is now ample evidence that cannabinoids and other GPCR agonists can modulate the development of Th cells. In the case of cannabinoids, it is possible that CB1 and CB2 may be differentially expressed on different subpopulations of APCs and Th cells⁵⁷. This selective expression could lead to an increase in Th2 development and a decrease in the development of Th1 cells, resulting in suppression of innate immunity and increased adaptive immunity⁴⁹. Continued research on the distribution and function of cannabinoid receptors as well as the production of endocannabinoids by different immune cells will provide greater insight into these mechanisms. One of the objective of the future studies should be not only to evaluate the therapeutic potential of

cannabinoids but also innate immune suppression brought about through cell biasing

D) CANNABINOIDS HAVE AN APOPTOTIC EFFECTS ON IMMUNE CELL

POPULATIONS: One major mechanism of immune suppression by cannabinoids is the induction of cell death or apoptosis⁵⁰. Apoptosis is defined as the programmed cell death caused by both intrinsic and extrinsic factors. Apoptosis eliminates damaged, harmful and unwanted cells, it is significant in biological processes including development, morphogenesis, and homeostasis. During apoptosis, many morphological changes occur such as membrane blebbing, cell shrinkage, mitochondria leakage, and nuclear fragmentation. Molecular changes underlie these morphological changes, and they make up the two different pathways of apoptosis: (1) intrinsic pathway – via mitochondria, and (2) extrinsic pathway – through death receptors.

Cannabinoids shows a wide range of apoptotic effect on different types of immune cells like B cells, T cells, antigen presenting cells like (Dendritic cells) and the malignant immune cells. In T cells prior studies have shown suppression of T cell proliferation. The endogenous cannabinoids have shown to induce apoptosis in the T cell culture. This was mostly brought about by the BCL-2 and the caspase activity. Systemic administration of THC has shown

suppressed proliferation of B cells, T cells and macrophages in spleen. CBD has shown apoptosis of CD4/ and CD8/ in a time and dose dependent manner.

Although the knowledge about the cannabinoids and their effect on innate response in gingival epithelial cells in context to oral cavity is limited. Few prior studies have shown that cannabinoid receptors especially CB1 play an important role in suppressing innate immunity through the TLR pathways utilizing the endocannabinoid ligands⁵⁸. Studies have also shown consistent expression of CB2 receptors in the gingival epithelial cells and the periodontal ligament cells⁵⁹,⁶⁰. Multiple studies have established marijuana as an important risk factor for chronic periodontitis in humans^{61, 62}. Moreover marijuana use appears to predispose teenagers and young adults to alveolar bone and attachment loss⁶³. Animal model studies have shown that cannabinoids also predispose animals to destructive periodontal disease. Rodents with ligated teeth exposed to marijuana smoke for only eight mins per day develop more extensive alveolar bone loss than ligated, unexposed animals⁶⁴, while reduced bone healing around implants has also been demonstrated in animals⁶⁵.

In conclusion we see that activation of CB2 may have an anti-inflammatory and anti-resorptive effects on LPS and *P. gingivalis*-stimulated TIGK cells. These findings suggest that activation of CB2 might be an effective therapeutic strategy for the treatment of

inflammation and alveolar bone resorption in periodontitis. Our study provides a potential rationale for the use of exogenous factors such as CB2-specific agonists as drugs in the treatment of periodontitis.

Further studies are required to corroborate the effect of CB2 activation on periodontitis in vivo.

TELOMERASE IMMORTALIZED GINGIVAL KERATINOCYTES (TIGKS)

Gingival keratinocytes are used in model systems to investigate the interaction between periodontal bacteria and the epithelium in the initial stages of the periodontal disease process. The gingival epithelium usually classified into sulcular epithelium, junctional epithelium (nonkeratinized) and oral gingival epithelium (keratinized). The junctional epithelium lies in the sulcus between the soft and hard tissues and has a unique structure. The junctional epithelium has intercellular spaces that create spaces for the immune cells to migrate into the gingival sulcus. They are the first to encounter the colonizing bacteria in the oral cavity and are thereby, wellknown to play an important role in the host bacterial environment. Several studies have shown the immunological modulation brought about by the epithelial cells the first line of defense is essential for the initiation and progression of periodontitis. Primary gingival epithelial cells have their limitations. Primary gingival epithelial cells are obtained from the gingival tissue of

healthy human subjects and then isolated and cultured in the laboratory .Primary gingival epithelial cells have been used as model systems for several studies. However, the cells have a finite lifespan and cease to grow after nine passages approximately and are then challenged to senescence and stop replicating any further. The limited life span poses a huge disadvantage in their usage. Also, there can be dissimilarities in their phenotype from donor to donor, questioning their use in in vitro experiments. To overcome the issue of finite life span, epithelial cells immortalized with viral proteins also known as the telomerase-immortalized human gingival epithelial cell line exhibited the capacity to undergo several passages. Thus a cell line which was comparable to the in vitro behavior of human GEC was developed⁶⁶.

Thus, we can summarize that with the literature supporting the use of cannabis as a potent anti-inflammatory agent over several decades in chronic inflammatory conditions, it would be interesting to find out the nature and extent of cannabinoid-induced immune suppression in epithelial cells and determine the mechanistic basis for this effect.

While clearly relevant to periodontal disease, such innate suppression is likely to be a central mediator of inflammation per se and, thus, have implications far beyond the oral cavity.

CHAPTER 2: MATERIALS AND METHODS

BACTERIAL CULTURE

Porphyromonas gingivalis ATCC 33277, *Filifactor alocis* ATCC 35896 and *Treponema denticola* ATCC 35405 were 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). *F. alocis* was grown in brain heart infusion (BHI) medium (Becton Dickinson, Sparks, MD) supplemented with L-cysteine (0.1%) and arginine (20%, both Sigma-Aldrich, St. Louis, MO). *T. denticola* was grown in tryptone-yeast extract-gelatin-volatile fatty acid-heat inactivated rabbit serum (TYGVS) medium. A broth medium had the following composition (per liter): tryptone (Difco Laboratories, Detroit, Mich.), 10.0 g; brain heart infusion broth, 5.0 g; yeast extract, 10.0 g; gelatin, 10.0 g; (NH₄)₂SO₄, 0.5 g; MgSO₄·7H₂O, 0.1 g; K₂HPO₄, 1.13 g; KH₂PO₄, 0.9 g; and NaCl, 1.0 g (TYGVS medium). The pH was adjusted to 7.2 with 4 N KOH before the autoclaving of the medium. The following ingredients were mixed, neutralized with KOH, sterilized by filtration, and added aseptically

to the autoclaved medium containing (final concentration, per liter): glucose, 1.0 g; cysteine hydrochloride, 1.0 g; thiamine pyrophosphate, 0.0125 g; sodium pyruvate, 0.25 g; acetic acid, 0.27 ml; propionic acid, 0.10 ml; n-butyric acid, 0.064 ml; n-valeric acid, 0.016 ml; isobutyric acid, 0.016 ml; isovaleric acid, 0.016 ml; DL-methylbutyric acid, 0.016 ml; and heat-inactivated rabbit serum, 10% (vol/vol). *P. gingivalis*, *F. alocis*, and *T. denticola* were grown under anaerobic conditions (80% N₂, 10% H₂, and 10% CO₂) at 37⁰c. Growth was monitored by tracking optical density at a wavelength of 600 nm. *T. denticola* ASLM, which had been isolated from human subgingival plaque, was maintained and grown at 34°C in an atmosphere of 85% N₂, 10% H₂, and 5% CO₂ in an anaerobic chamber (Coy Manufacturing, Ann Arbor, Mich.) in a broth medium which had the following composition (per liter): tryptone (Difco Laboratories, Detroit, Mich.), 10.0 g; brain heart infusion broth, 5.0 g; yeast extract, 10.0 g; gelatin, 10.0 g; (NH₄)₂SO₄, 0.5 g; MgSO₄·7H₂O, 0.1 g; K₂HPO₄, 1.13 g; KH₂PO₄, 0.9 g; and NaCl, 1.0 g (TYGVS medium). The pH was adjusted to 7.2 with 4 N KOH before the autoclaving of the medium. The following ingredients were mixed, neutralized with KOH, sterilized by filtration, and added aseptically to the autoclaved medium containing (final concentration, per liter): glucose, 1.0 g; cysteine hydrochloride, 1.0 g; thiamine pyrophosphate, 0.0125 g; sodium pyruvate, 0.25 g; acetic acid, 0.27

ml; propionic acid, 0.10 ml; n-butyric acid, 0.064 ml; n-valeric acid, 0.016 ml; isobutyric acid, 0.016 ml; isovaleric acid, 0.016 ml; DLmethylbutyric acid, 0.016 ml; and heat-inactivated rabbit serum, 10% (vol/vol).

CELL CULTURE

Telomerase immortalized gingival keratinocytes (TIGKs) were maintained as frozen stocks and cultured in Dermalife-K serum free keratinocyte culture medium (Lifeline cell technology, Walkersville, MD) containing the supplements, transferrin 5ug/ml, rh TGF- α 0.5ng/ml, rh insulin 5ug/ml, epinephrine 1.0uM, L- glutamine 6mM, hydrocortisone 100ng/ml and extract-P 0.4%⁶⁶. Cells were grown in a humidified chamber at 37°C, 5% CO₂. Medium was changed every 2-3 days and cells were passaged when 80-90% confluent. Cells were passaged by washing twice with phosphate-buffered saline (PBS) and incubating with 0.05% Trypsin/0.53mM EDTA at 37°C for 5-10 minutes until cells lifted from the surface of the flask. Dermalife-K serum free keratinocyte culture medium was added to the cell suspension to inhibit the enzymatic activity of trypsin/EDTA, and cells were centrifuged at 9.7 g for 10 minutes. The supernatants were removed, the cell pellet re-suspended in medium and re-seeded at 1.0×10^6 cells/T75 flask. The cells were seeded into 6well plates at 30% confluence and infected when 80% confluence was attained (approx. 1.0×10^6 cells/well).

CYTOKINE EXPRESSIONS PATTERNS IN GINGIVAL EPITHELIAL CELLS

TIGK cells were exposed, or not, to three cannabinoid subtypes [cannabidiol, cannabinal, or tetrahydrocannabinol; 1, 5 or 10 µg /ml] and epithelial viability was determined using the Trypan blue exclusion test. TIGKs (1×10^6 cells per well) were also challenged, or not, with *P. gingivalis* at MOI of 10 in the presence or absence of cannabinoids [cannabidiol, cannabinal, or tetrahydrocannabinol; 0, 1, or 5 µg /ml]. Bacterial cells were harvested at mid to late log phase. Cytokine (IL-6, IL-8 and IL-10) induction and release was determined by ELISA, according to the manufacturer's instructions. TIGKs (1×10^6 cells per well) were also challenged with LPS at concentration of 1µg/ml in the presence or absence of cannabinoids [cannabidiol, cannabinal, or tetrahydrocannabinol; 0, 1, or 5 µg /ml]. Cytokine (IL-6, IL-8 and IL-10) induction and release was again determined by ELISA, according to the manufacturer's instructions. To check for the functional significance of the CB2 receptor, a CB2-specific pharmacological inhibitor, JTE907 (Tocris, Bioscience), was used at concentration of 5 µg/ml. Cells were exposed for 30 min to

the inhibitor then exposed to cannabinoid subtypes for 2 hours and then exposed to of bacterial stimulant LPS 1ug/ml and *P. gingivalis* at MOI of 10:1 and incubated overnight. Twenty hour cell-free supernatants were collected by centrifugation at 9.7 x *g* for 4 mins. All experiments were performed in triplicate.

STATISTICAL ANALYSIS

Statistical analyses were performed using Graph Pad Prism 6 (Graph Pad Software, San Diego, CA). Data are presented as mean, standard deviation of the mean (SD). Differences were analyzed with one-way ANOVA and Student t test, as appropriate. Statistical significance was set at $p < 0.05$.

EJ CRVGT'5"RESULTS

3.1 Cannabinoids, *P. gingivalis* and TIGK cell viability

HIGH CONCENTRATIONS OF CANNABINOIDS ARE LETHAL TO TIGK CELLS

To investigate the influence of three archetypal cannabinoids on TIGK viability, we exposed TIGK cells to various concentrations of cannabidiol, cannabinol and tetrahydrocannabinol. As shown in **Figure 1**, each cannabinoid subtype significantly lowered TIGK viability at concentrations of 10 $\mu\text{g/ml}$, as determined by Trypan blue exclusion. However, TIGK viability was not influenced by cannabinoid concentrations of 5 $\mu\text{g/ml}$ or less.

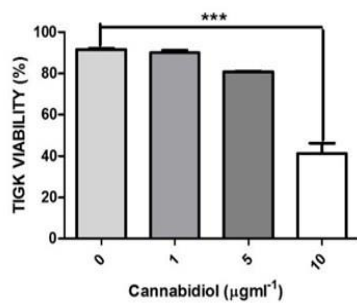


Figure 1A

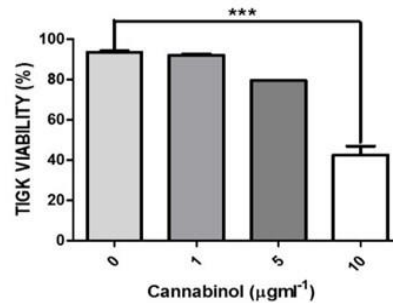


Figure 1B

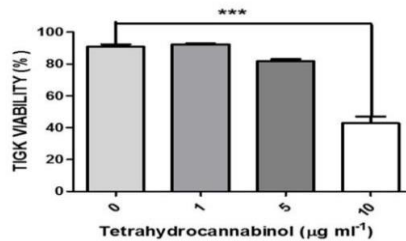


Figure 1C

FIGURE 1: Viability of cannabinoid-exposed TIGK cells.

TIGK viability following exposure to (A) CBD, (B) CBN or (C) THC, at the cannabinoid concentrations noted, was determined by Trypan blue exclusion.

*** $p < 0.001$ compared to the untreated control.

P. gingivalis DOESNOT ALTER VIABILITY OF
CANNABINOID EXPOSED TIGK CELLS

The viability of cannabinoid-exposed and control TIGKs were also monitored in presence and absence of *P. gingivalis* (MOI 10:1), again, by Trypan blue cell exclusion assay. The data presented in **Figure 2** confirm that each cannabinoid subtype significantly suppressed TIGK viability at concentrations of 10 $\mu\text{g/ml}$, while TIGK viability was not influenced by cannabinoid concentrations of 5 $\mu\text{g/ml}$ or less. *P. gingivalis* did not influence TIGK viability in the presence or absence of cannabinoids. Therefore, 0-5 $\mu\text{g/ml}$ cannabinoid dosing regimens and bacterial infections at MOI 10:1 were employed in the remainder of the experiments.

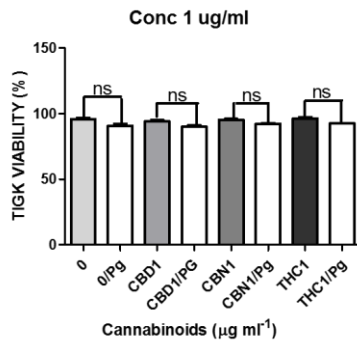


Figure 2A

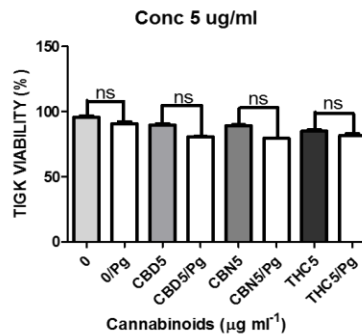


Figure 2B

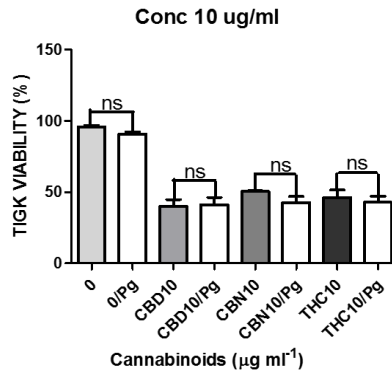


Figure 2C

FIGURE 2: *P. gingivalis* does not influence the viability of cannabinoid-exposed TIGK cells.

TIGK viability following exposure to CBD, CBN or THC at (A) 1 µg/ml, (B) 5 µg/ml or (C) 10 µg/ml, was determined by Trypan blue exclusion. *P. gingivalis* did not influence the viability of cannabinoid-exposed TIGK cells (all, $p > 0.05$ compared to the untreated control).

3.2 Cannabinoid subtypes and the production of pro-inflammatory

cytokines in LPS & *P. gingivalis*-stimulated TIGK cells.

CANNABINOIDS SUPPRESS THE PRODUCTION OF IL-8 IN LPS & *P.*

gingivalis-STIMULATED TIGK CELLS

Bacterial lipopolysaccharides (LPS) are major outer surface membrane components present in almost all Gram-negative bacteria⁶⁷. The lipid A portion of LPS is a potent stimulator of innate immunity^{67, 68}. Escherichia coli-derived LPS represents the classic TLR4-dependent proinflammatory agonist^{68, 69}. IL-8 is a major pro-inflammatory chemokine that is released by epithelial cells that have detected microbial signals that, among other functions, acts as a chemoattractant to immune cells, particularly neutrophils^{70, 71}. *P. gingivalis* is a major periodontal pathogen⁷². It causes perturbation of the epithelial cells, invades the periodontal tissue and evades host defense mechanisms⁷². In doing so, it utilizes its panel of virulence factors causing deregulation of the innate immune response and inflammatory process^{72, 73}. *P. gingivalis* exists commensally in the host and causes a dysbiotic shift of the microbial environment during

the disease episodes¹⁷. It triggers a wide array of changes in the gingival epithelial cells such as selective inhibition of certain immune mediators and modulation of signaling pathways causing

dysregulation of innate immune response⁷⁴. To check the influence of marijuana derived cannabinoid subtypes on the release of IL-8, we exposed TIGK cells to cannabinoid subtypes followed by LPS or *P. gingivalis*. As shown in **Figure 3**, cannabinoids (CBD, CBN or THC; 1 and 5 $\mu\text{g} / \text{ml}$) significantly inhibited the LPS and *P. gingivalis*-induced IL-8 response in TIGK cells.

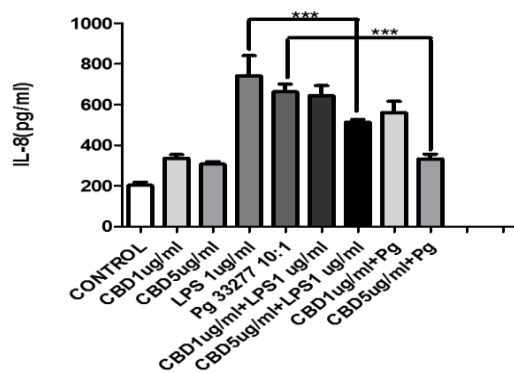


Figure3A

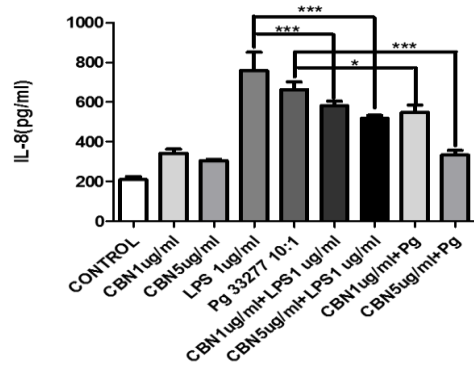


Figure 3B

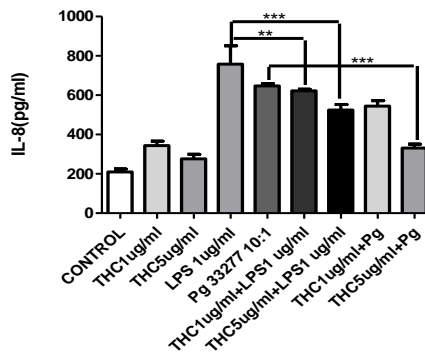


Figure 3C

FIGURE 3: Cannabinoids suppress IL-8 release in LPS & *P. gingivalis*-stimulated TIGK cells. (A) CBD, (B) CBN or (C) THC, at the concentrations noted, suppressed IL-8 release in LPS (1 µg/ml)-stimulated TIGK cells, as determined in 20 hr cell-free supernatants by ELISA. TIGK cells were exposed to cannabinoid subtypes (1 & 5 µg/ml) for 2 hr. followed by addition of LPS (0-1 µg/ml) or *P. gingivalis* [MOI 10:1] for 18 hr. Figure 3, shows stats significant variation from *P. gingivalis* above, however LPS also shows significant difference from all points on the graph except for CBD1 µg/ml +LPS (1 µg/ml) & *P. gingivalis* alone (Figure 3A); *P. gingivalis* alone (Figure 3B). */ *** p < 0.5 / 0.001, respectively, compared to the *P. gingivalis* control.

CANNABINOIDS SUPPRESS PRODUCTION OF IL-6 IN LPS & *P.*

gingivalis- STIMULATED TIGK CELLS

Periodontal pathogens and bacterial-derived stimulants, including LPS, initiate pro-inflammatory changes in the periodontium cytokine production^{75, 76}. In the gingiva, IL-6 is produced by mononuclear cells. These Macrophages were shown to secrete IL-6 in response to LPS in a dose-dependent manner⁷⁷. The association between IL-6 and alveolar bone loss in periodontal disease was also established in animal models⁷⁸. IL-6, synergistic with other inflammatory cytokine induces bone resorption^{78, 79}. Having established that cannabinoids suppress the IL-8 response of TIGK cells, we next examined the influence of CBD, CBN and THC on LPS or *P. gingivalis* induced secretion of IL-6 from TIGK cells. As shown in **Figure 4**, cannabinoids, (CBD, CBN or THC; 1 and 5 µg / ml) significantly inhibited the LPS & *P. gingivalis*-induced IL-6 response in TIGK cells in a similar manner to IL-8.

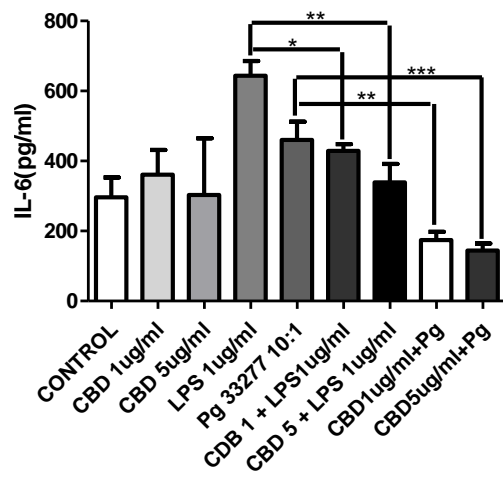


Figure 4A

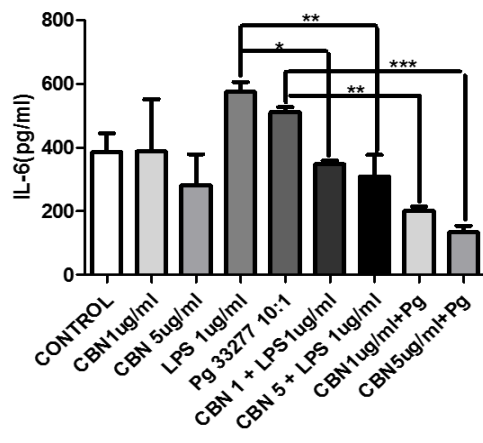


Figure 4B

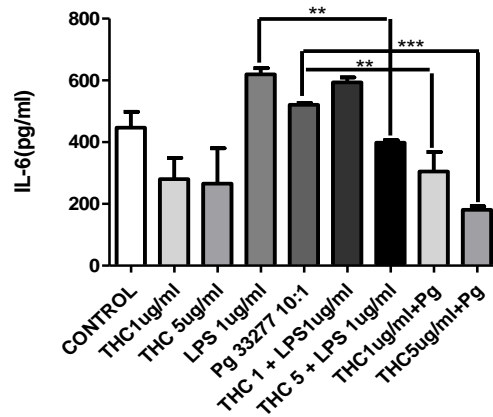


Figure 4C

FIGURE 4: Cannabinoids suppress IL-6 release in LPS & *P. gingivalis*-stimulated TIGK cells. (A) CBD, (B) CBN or (C) THC, at the concentrations noted, suppressed IL-6 release in LPS (1µg/ml)-stimulated TIGK cells, as determined in 20 hr cell-free supernatants by ELISA. TIGK cells were exposed to marijuana derived cannabinoid subtypes at (1 & 5µg/ml) for 2hr, followed by LPS(0-1µg/ml) or *P. gingivalis* [MOI 10:1] for 18hr. Figure 4, shows stats significant variation from *P. gingivalis* above, however LPS also shows significant difference from all points on the graph except for *P. gingivalis* alone (Figure 4A); CBN 1µg/ml and *P. gingivalis* alone (Figure 4B); THC 1µg/ml + LPS (1µg/ml) (Figure 4C). **/ *** p < 0.01 / 0.001, respectively, compared to the *P. gingivalis* control.

Inflammatory cytokine ,IL-10, in LPS & P. gingivalis-stimulated

TIGK cells

CANNABINOIDS ENHANCE THE PRODUCTION OF IL-10 IN IN

LPS & P. gingivalis STIMULATED TIGK CELLS

Bacterial stimuli-induced cytokines play a major role in the progression of chronic periodontitis⁸⁰. Earlier, we considered the pro-inflammatory cytokines, IL-6 and IL-8. However, it is the balance of pro- and anti-inflammatory mediators that determine the severity, duration and direction of innate responses⁸¹. IL-10 is a key anti-inflammatory cytokine that has been shown to have osteoprotective properties⁸². *P. gingivalis* is known to be a major periodontal pathogen associated with chronic periodontitis. IL-10 is an anti-inflammatory cytokine known to have an important function in balancing the effects of the pro-inflammatory cytokines, thus modulating the progression of the chronic inflammation and exacerbation of the disease state⁸¹. Therefore, we next determined the influence of marijuana derived cannabinoid subtypes on the release of IL-10 from LPS and *P. gingivalis*-stimulated TIGK cells. As shown in **Figure 5** cannabinoids (CBD, CBN or THC; 1 and 5 µg / ml) significantly enhanced the LPS and *P. gingivalis* induced IL-10 response in TIGK cells.

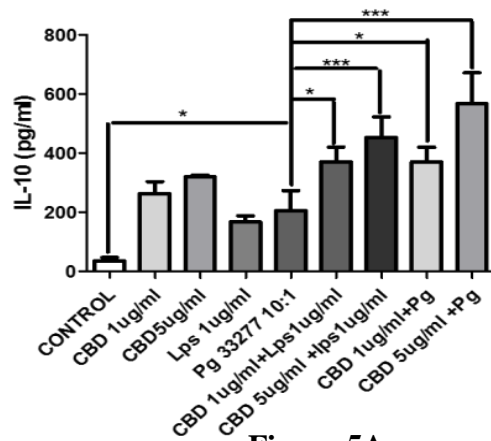


Figure 5A

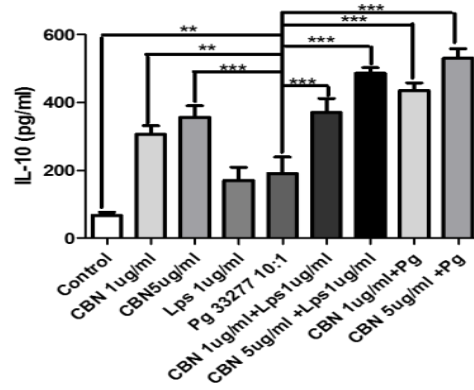


Figure 5B

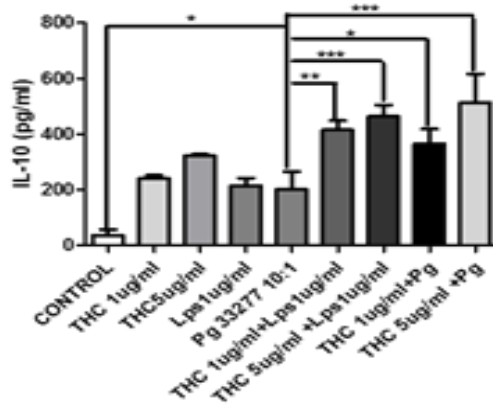


Figure 5C

FIGURE 5: Cannabinoids enhance IL-10 release in LPS & *P. gingivalis*-stimulated TIGK cells.

(A) CBD, (B) CBN or (C) THC, at the concentrations noted, enhanced IL-10 release in LPS (1 μ g/ml)-stimulated TIGK cells, as determined in 20 hr cell-free supernatants by ELISA. TIGK cells were exposed to marijuana derived cannabinoid subtypes at (1 & 5 μ g/ml) for 2hr, followed by LPS (0-1 μ g/ml) or *P. gingivalis* [MOI 10:1] for 18hr. Figure 5, shows stats significant variation from *P. gingivalis* above, however LPS also shows significant difference from all points on the graph except for CBD 1 μ g/ml, CBD 5 μ g/ml, negative control & *P. gingivalis* (Figure 5A); *P. gingivalis* (Figure 5B). THC 1 μ g/ml, THC 5 μ g/ml and *P. gingivalis* (Figure 5C). **/ *** $p < 0.01 / 0.001$, respectively, compared to the *P. gingivalis* control.

3.6 Pharmacological inhibition of CB2

THE CB2 INHIBITOR, JTE 907, FAILS TO RESCUE THE PRODUCTION OF IL-8 or IL-6 IN TIGK CELLS

We hypothesized that the anti-inflammatory effect of the cannabinoid subtypes are mediated through epithelial CB2 receptors. In order to confirm this hypothesis, we used a specific pharmacological inhibitor of CB2, JTE907⁸³, at a concentration of 5µg/ml. TIGK cells were pretreated with JTE907 (5µg/ml) for 30min. Therefore, we next determined the influence of marijuana derived cannabinoid subtypes on the release of pro-inflammatory cytokine IL-8 and IL6 from *P. gingivalis*-stimulated TIGK cells pretreated with JTE 907. As shown in **Figures 6** and **7**, JTE907 failed to rescue IL-8 production in LPS- or *P. gingivalis* stimulated TIGKs. Similarly, as shown in **Figures 8** and **9**, JTE907, failed to rescue IL-6 production in LPS- or *P. gingivalis*stimulated TIGKs. These data imply that CB2 is not the surface receptor responsible for cannabinoid initiated anti-inflammatory signals in gingival epithelial cells.

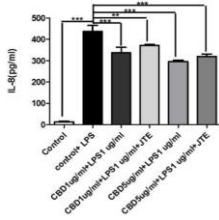


Figure 6A

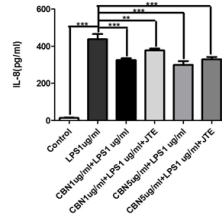


Figure 6B

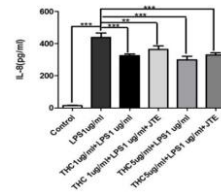


Figure6C

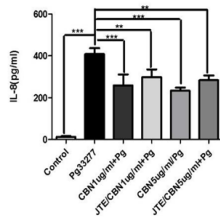


Figure 7A

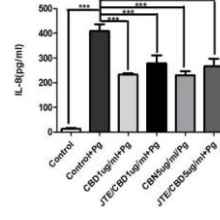


Figure 7B

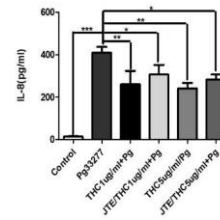


Figure 7C

FIGURES 6 and 7: Pretreatment with a specific CB2 inhibitor fails to rescue IL-8 release in *P. gingivalis*- and LPS -stimulated TIGK cells.

(A) CBD, (B) CBN or (C) THC, at the concentrations noted, suppressed IL-8 release in (Figure 9) LPS- and (Figure 10) *P. gingivalis*-stimulated TIGK cells. IL-8 production in TIGK cells pretreated with the CB2 inhibitor, JTE907 (5 µg/ml 30 mins), was also determined. TIGK cells were exposed to JTE-907 for 30 mins followed by cannabinoid subtypes (1& 5 µg/ml) for 2hr and then to LPS (1µg/ml) or *P. gingivalis* (MOI of 10:1) for 18hr. **/ *** p < 0.01 / 0.001, respectively, compared to the LPS (Figure 9) or *P. gingivalis* only controls.

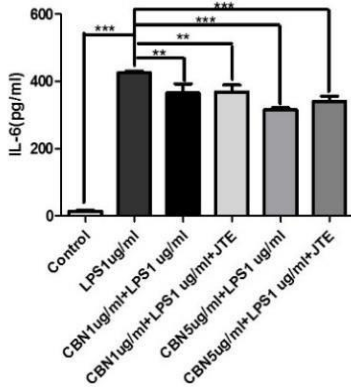


Figure 8A

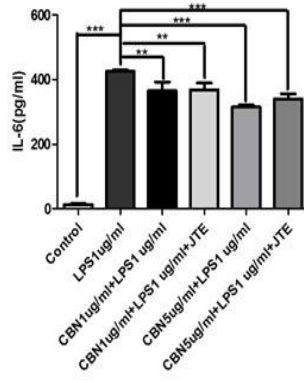


Figure 8B

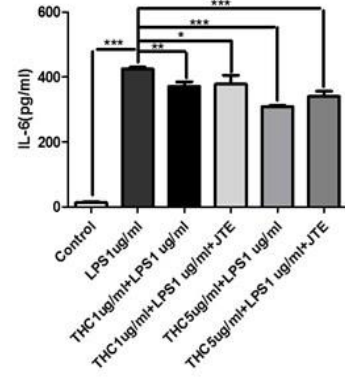


Figure 8C

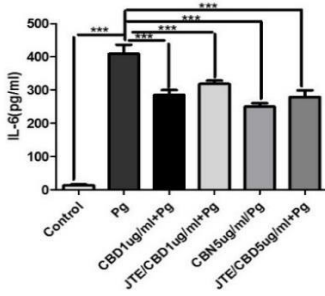


Figure 9A

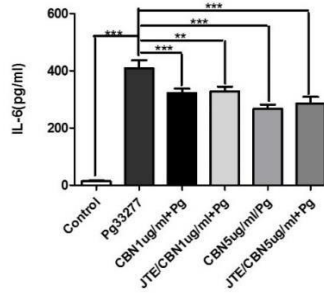


Figure 9B

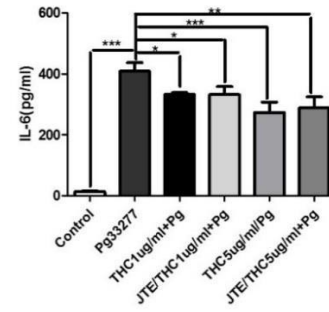


Figure 9C

FIGURES 8 and 9: Pretreatment with a specific CB2 inhibitor fails to rescue IL-6 release in *P. gingivalis*- and LPS -stimulated TIGK cells.

(A) CBD, (B) CBN or (C) THC, at the concentrations noted, suppressed IL-6 release in (Figure 11) LPS- and (Figure 12) *P. gingivalis*-stimulated TIGK cells. IL-6 production in TIGK cells pretreated with the CB2 inhibitor, JTE907 (5 $\mu\text{g/ml}$ 30 mins), was also determined. TIGK cells were exposed to JTE-907 for 30 mins followed by cannabinoid subtypes (1 & 5 $\mu\text{g/ml}$) for 2hr and then to LPS (1 $\mu\text{g/ml}$) or *P. gingivalis* (MOI of 10:1) for 18hr.*/**/ *** p < 0.05 / 0.01 / 0.001, respectively, compared to the LPS (Figure 9) or *P. gingivalis* only controls.

3.7 The influence of cannabanoids on the growth of select oral pathogens

HIGH DOSES OF CANNABINOIDS SUPPRESS THE GROWTH OF *P.GINGIVALIS* AND *F. ALOCIS* BUT NOT *T. DENTICOLA*

To check the effect of cannabinoid subtypes on the viability of the major representative oral periodontal pathogens like *P. gingivalis*, *F. alocis* & *T. denticola*. We exposed these bacterial cultures to the noted concentrations of the cannabinoid subtypes and plotted the growth bacteria was grown in their respective culture medium. *P. gingivalis* was grown in GAM supplemented, or not, with cannabinoid subtypes cannabiniol, cannabidiol and tetrahydrocannabinol at concentrations of 0.1, 1, 5 & 10 µg/ml and growth monitored by absorbance measurements at O.D. 600 nm. As shown in **Figure 10**, higher doses of cannabinoids (CBD, CBN or THC) inhibited the growth of *P. gingivalis*. Similar results were noted for *F. alocis* (see **Figure 11**), whereas *T.denticola* was resistant to all cannabinoid doses tested (see **Figure 12**).

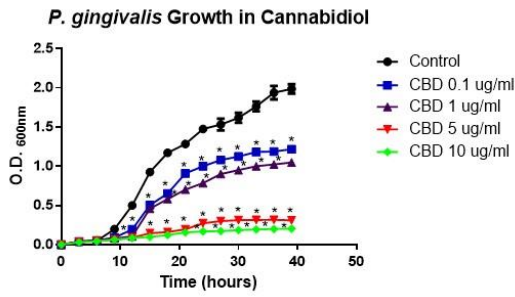


Figure 10A

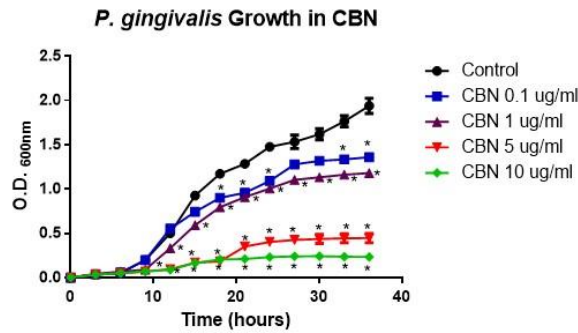


Figure 10B

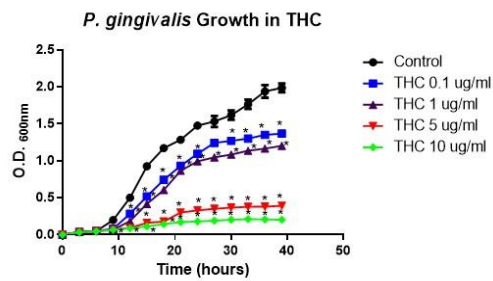


Figure 10 C

FIGURE 10: High dose cannabinoids suppress the growth of the periodontal pathogen, *P. gingivalis*. *P. gingivalis* was grown in GAM supplemented, or not, with (A) CBD, (B) CBN or (C) THC, at the concentrations noted, and growth monitored by absorbance measurements at O.D. 600 nm. */**/ *** $p < 0.05 / 0.01 / 0.001$, respectively, compared to control.

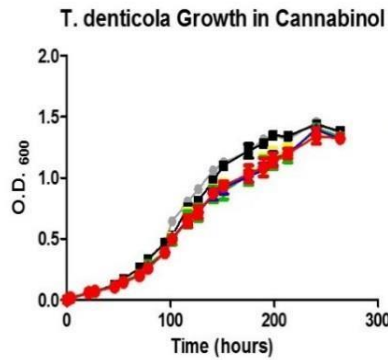


Figure 11A

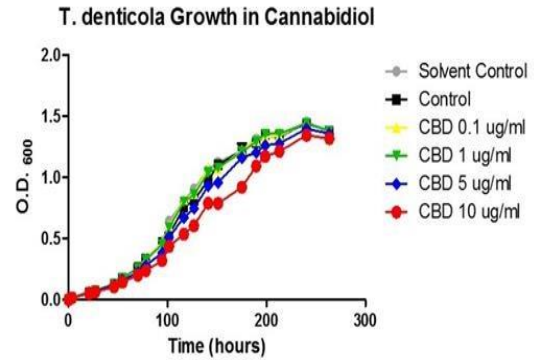


Figure 11B

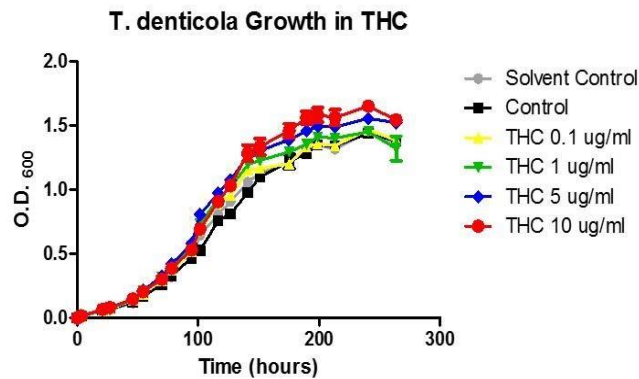


Figure 11C

FIGURE 14: Cannabinoids do not influence to growth of *T. denticola*.

T. denticola was grown in TYGVS medium supplemented, or not, with (A) CBD, (B) CBN or (C) THC, at the concentrations noted, and growth monitored by absorbance measurements at O.D. 600 nm. There were no statistically significant differences in the growth characteristics of cannabinoid -exposed and control bacteria (all, $p > 0.05$ compared to the untreated control).

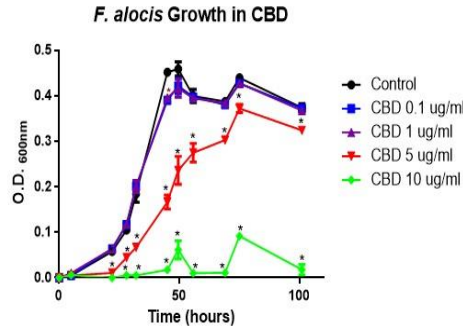


Figure 12A

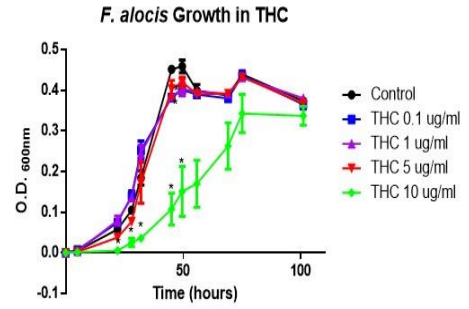


Figure 12B

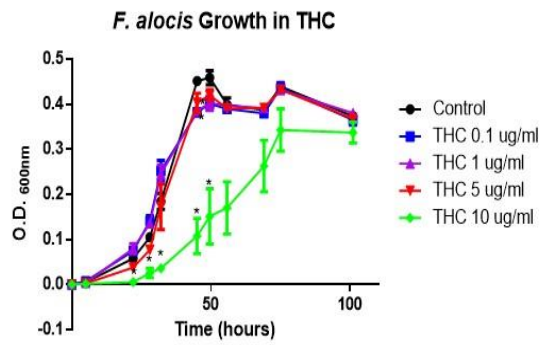


Figure 12C

FIGURE 12: High dose cannabinoids suppress the growth of the periodontal pathogen, *F. alocis*.

F. alocis was grown in BHI supplemented, or not, with (A) CBD, (B) CBN or (C) THC, at the concentrations noted, and growth monitored by absorbance measurements at O.D. 600 nm.

*/ *** $p < 0.05 / 0.001$, respectively, compared to control

CHAPTER 4 DISCUSSION

Cannabis use has been etiologically associated with periodontal disease susceptibility, but the underlying mechanisms are unknown⁶¹,⁶². We have established that (i) high doses of marijuana derived cannabinoids negatively influence the viability of TIGK cells; (ii) sub-toxic doses of cannabinoids suppress pro-inflammatory cytokine production by TIGK cells responding to LPS or to *P. gingivalis*; (iii) sub-toxic doses of cannabinoids enhance anti-inflammatory IL-10 production by TIGK cells responding to LPS or to *P. gingivalis*; (iv) a normal innate response to bacterial stimuli is not rescued by pharmacological inhibition of CB2 in TIGK cells; and that (v) high doses of cannabinoids inhibit the growth of *P. gingivalis* and *F. alocis* but not *T. denticola*.

Should these five phenomena occur in the oral cavity of human cannabis users, then we start to explain how marijuana, specifically marijuana-derived cannabinoids, may predispose to chronic periodontitis and/ or other periodontal diseases.

The oral epithelial barrier separates the host from the environment and provides the first line of defense against pathogens, exogenous substances and mechanical insults⁸⁴. It consists of underlying connective tissue and a stratified keratinized epithelium with at membrane, whose cells undergo terminal differentiation resulting in

the formation of a mechanically resistant surface. Gingival keratinocytes are connected by various transmembrane proteins, such as tight junctions, adherens junctions and gap junctions, each of which has a specialized structure and specific functions⁸⁴. Periodontal pathogens are able to induce inflammatory responses that lead to attachment loss and periodontal destruction. A number of studies have demonstrated that the pathogenic oral bacteria influence the expression and structural integrity of different cell-cell junctions³⁷. Tissue destruction can be mediated by host cells following stimulation with cytokines and bacterial products⁷⁵. To summarize, the oral epithelium is able to react to a variety of exogenous, possibly noxious influences.

Thus an intact oral epithelial barrier is a key component of the host oral defense system. In consonance to the findings in our study, we observe that the higher concentrations of marijuana derived cannabinoid subtypes proved to have a negative effect on gingival epithelial cell viability hence compromising the intactness of the epithelial layer required for the initial host defense to periodontal pathogens. It is possible that cannabinoid induced epithelial cell death may compromise the integrity of this junctional epithelium layer, facilitating breach by periodontal pathogens and, more importantly, by bacterial-derived toxins known to contribute to the disease process

in chronic periodontitis. This may explain the predisposition of chronic periodontitis in marijuana users.

As we know, the etiology of periodontal diseases is bacterial. The human oral cavity harbors an evolving load of microbial species. The ecological interactions between the host and oral microbes determine the severity of the disease process. The critical interplay between the host immune mechanisms and inflammatory responses determine the pathogenesis of complex diseases like chronic periodontitis⁷⁰. The immunological events seem to overlap during different phases of the disease thus making the understanding of the pathogenesis of the disease difficult.

The metabolic products of bacteria trigger junctional epithelium cells to produce cytokines. The acute inflammation, is the physiologic response to the microbial challenge to recruit adequate immune cells to site of infection through the production of cytokines and chemokines^{85, 86}. Innate immunity is usually, characterized by the phagocytosis and digestion of microorganisms and foreign substances by macrophages and neutrophils⁸⁶. Phagocytes such as macrophages and neutrophils have surface receptors that recognize and bind surface molecules of bacteria⁸⁶. Thus these cytokines are seen to play a protective role by helping to recruit immune cells to the site of the inflammation^{87, 88}. The host responds to microbial challenge by generating an inflammatory cell infiltrate in the tissue subjacent to the

periodontal pocket⁷⁰. The cytokines and chemokines form an integral part of the initial innate immune response, they signal the submucosal effector molecules leading to the activation of inflammatory signaling pathways like NF- κ B⁸⁹. Cytokines are produced by resident cells, such as epithelial cells and fibroblasts, and by phagocytes (neutrophils and macrophages) in the acute and early chronic phases of inflammation⁷⁰.

The first cytokine identified to have chemotactic activity was interleukin-8. In the periodontium, this cytokine is produced primarily by gingival fibroblasts, gingival epithelial cells and endothelial cells⁹⁰. Interleukin-8 is a polymorphonuclear leukocyte chemoattractant⁷¹. It is detectable in healthy and diseased periodontal tissues and has been associated with subclinical inflammation of the initial phase of the disease which is comprised of polymorphonuclear neutrophil gradient along the intact junctional epithelium. Prior studies have established that *P. gingivalis* evade and disrupt the host immune homeostasis contributing to the exacerbation of the disease process in chronic periodontitis⁹¹. It has also been established that *P. gingivalis* produce gingipains a potent virulence factor which causes inactivation of pro-inflammatory cytokine IL-8⁹². This implies that the periodontal pathogen survives longer evading the immune response, contributing to polymicrobial dysbiosis of the periodontal community¹⁷. Interleukin-6 is another pro-inflammatory cytokine

which is one of the first few cytokines to appear in the periodontal disease pathogenesis and is known to play an important part in the progression of the chronic periodontitis. Interleukin-6 is an innate cytokine which has been associated with inflammatory cell migration and osteoclastogenesis in chronic periodontitis⁹³.

In accordance to the prior studies, in our study we observe that the marijuana derived cannabinoid subtypes abrogate production of pro-inflammatory cytokine IL-8 and IL-6 from LPS or *P. gingivalis* stimulated TIGK cells. It may imply that the abrogation of these cytokines would dysregulate the first line of innate immune response in the epithelial cells. Cells pertaining to innate immunity like neutrophils and macrophages would no longer be able infiltrate at the site of inflammation. Hence the periodontal pathogen would be able to survive for a longer period of time and secrete potent proteolytic enzymes like proteases which would further accentuate the destruction of the periodontium. The result of our study confirms the well-established anti-inflammatory property of cannabinoids and how it supports the suppression of the innate immune response explaining the increasing susceptibility of marijuana users to chronic periodontitis. Furthermore, suppression of inflammation, if it also occurs *in vivo*, implies that clinicians may need to increase screening vigilance in order to detect the onset of periodontal tissue destruction in cannabis smokers with a suppression of clinical signs of

inflammation with response down regulation of pro-inflammatory cytokines.

The role of pro-inflammatory cytokines in the periodontal tissue destruction is well documented in the animal model⁹⁴. However, cellular responses to the pro-inflammatory cytokines are regulated negatively by anti-inflammatory cytokines and the balance between these two categories of cytokine could be important in the outcome of inflammatory response⁸¹. The prior studies showed that anti-inflammatory cytokine IL-10 was suppressed by pro-inflammatory cytokine in chronic periodontitis⁹⁵. The studies also showed that the difference of pro and anti-inflammatory cytokine (IL-10) in periodontitis was very subtle but significant to cause destruction of the periodontal structures in chronic periodontitis⁸¹. It was noted in our study that there was concomitant rise in the expression of anti-inflammatory cytokine IL-10 from LPS/ *P. gingivalis* stimulated TIGK cells when exposed to marijuana derived cannabinoid subtypes at sub-lethal doses. The findings of our study does not seem to be in accordance to the prior studies .The elevated levels of IL-10 on exposure to cannabinoids in epithelial cells implies that marijuana derived cannabinoids although are implicated as a risk factor for chronic periodontitis they suppress pro-inflammatory cytokine and increases the anti-inflammatory ones which is opposite to the pattern usually observed in earlier studies involving chronic periodontitis⁹⁵.It

is possible that cannabinoid induced suppression of pro-inflammatory cytokines may prevent the recruitment of immune cells like neutrophils which is a spontaneous reaction of the innate immune mechanism and up regulation of IL-10 further contributes in suppressing the pro-inflammatory cytokines (cellular crosstalk) preventing the initial innate inflammatory response. Thus helping the disease associated pathogen to evade the immune response. As cannabinoids are known to be anti-inflammatory it is possible that it plays a role in upregulating the expression of anti-inflammatory cytokine IL-10. It has been shown in prior studies that IL-10 and cannabinoids have an osteo-protective effect^{82, 96}. Therefore it is necessary to research further in this direction to gain a mechanistic insight that would explain the osteo-protective influence of cannabinoid subtypes in context to its anti-inflammatory property in chronic periodontitis model.

It has been noted previously that CB2 is expressed in the gingival epithelial cells comprising the junctional epithelium^{59, 60}. We had hypothesized that the anti-inflammatory actions of cannabinoids may be mediated through such surface CB2 receptors. It was noted that pharmacological inhibition of CB2 receptor did not rescue the innate immune response to bacterial stimulants in epithelial cells. Further research would be needed to understand the functional importance of the CB2 receptor. Other specific inhibitor like AM630, along with

siRNA-mediated CB2 gene silencing, could be used to determine the importance of CB2 in cannabinoid-mediated suppression of the innate response to dental plaque pathogens in human gingival epithelial cells. CB2 expression could be also studied using qPCR telling us more about the mRNA levels.

It is also possible that the JTE907 results are genuine, therefore the anti-inflammatory action of cannabinoids may be brought about by other non-CB2 target receptors. Therefore creating a scope of studying other potential target receptors like the CB1, GPR55 and A2A receptors which may be associated with the anti-inflammatory function of marijuana derived cannabinoids^{97,98}.

Finally, the findings that *P. gingivalis* and *F. alocis*, but not *T. denticola*, are susceptible to cannabinoids is both novel and intriguing. Essentially, clinically healthy periodontal tissue maintains a highly ordered, mild state of inflammation⁷⁰. It is usually established that the normal oral microflora, which is thought to provide the stimulus for this mild inflammatory response. However, the highly ordered state of mild inflammation is replaced by a disordered state of severe inflammation with the altered microbial community¹⁴. As seen that suppression and negative influence of marijuana derived cannabinoid subtypes on periodontal pathogen *P. gingivalis* and *F. alocis* seem to alter the periodontal microbial community. Thus, it is proposed that the shift from a symbiotic

microflora to a dysbiotic pathogenic community triggers the potent host inflammatory response that contributes to the tissue destruction and alveolar bone loss that are characteristic of periodontitis¹⁷.

There are a number of limitations to this study. It is possible that cytokine release in response to *E. coli* LPS, used as a positive control in much of the experiments herein, may have been optimized with the addition of serum as soluble CD14 is present in serum and facilitates binding of LPS. Binding of LPS to CD14 is enhanced by the presence of the LPS binding protein (LBP)⁹⁹. LBP may also enhance binding of whole Gram-negative bacteria to cells thus transferring LPS to a second receptor that directly transduces the signal thus or by the use of protease inhibitors, that would stabilize cytokines produced. Further, we did not monitor the activity of cytokine-encoding genes.

In the future, further research could be carried out to analyze the complex signaling pathways associated in cannabinoid-induced suppression of innate immunity in epithelial cells. The information generated would be further used in gingival epithelial cells, other innate cell types, and additional pathogen strains as well as the establishment of in vivo relevance in mouse models of periodontitis and inflammation.

In summary, cannabinoids exert multiple negative effects on epithelial viability, on epithelial responses to bacterial stimuli and on

bacterial growth characteristics. In concert, these events are likely to predispose individuals who smoke marijuana to periodontal diseases. Further delineation of marijuana-periodontal disease interactions may be warranted. Determination of the amount of cannabinoids found in the gingival crevice of cannabis users; the mechanisms of cannabinoid induced epithelial death; the signaling pathways controlling innate immune suppression; and the influence of cannabis intake on the periodontal microbiome are all likely to provide useful information helping to explain how marijuana induces and/or exacerbates periodontal diseases. Such insights are needed for the development of novel therapeutic and preventive approaches for this growing section of American society.

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