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Exploring a novel NF-κB- inhibiting nanoparticle for periodontitis therapy.

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EXPLORING A NOVEL NF-κB- INHIBITING NANOPARTICLE FOR PERIODONTITIS THERAPY

By

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A Thesis approved on

April 18, 2017

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DEDICATION

This thesis is dedicated to

My parents- USR Murthy and SK Devi

Brother- Soma Upadhyayula

Shanti Pittampalli

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The completion of this project required lot of guidance and assistance from many people and I am extremely fortunate to have excellent guidance and support from a lot of people whose names may not all be enumerated. Their contributions are sincerely appreciated and acknowledged.

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ABSTRACT

EXPLORING A NOVEL NF-κB- INHIBITING NANOPARTICLE FOR PERIODONTITIS THERAPY

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Periodontitis is an infection-driven inflammatory disease characterized by gingival inflammation and bone loss. The NF-κB signaling pathway is pivotal in osteoclastogenesis and infection-induced pro-inflammatory responses. The use of nanoparticles as a vehicle to deliver drug increases stability, loading capacity, and facilitates transmembrane transportation. The hypothesis was that a novel nanoparticle carrying therapeutic NBD inhibitory peptides (NBD-nanoparticles) will inhibit measures of periodontal disease. In this project, we tested the nanoparticles for their ability to directly inhibit osteoclastogenesis and inflammation as an original strategy for periodontitis therapy. We also tested the capability of the nanoparticles to inhibit gingival inflammation and alveolar bone loss in an animal model.

Methods: *In vitro*- In order to test the impact of NBD-nanoparticles on osteoclastogenesis, RAW 264.7 cells were stimulated using RANKL and treated with NBD-nanoparticles. Controls included treatment with empty nanoparticles and no treatment. Seven days later, the cultures were fixed and stained with

TRAP, an osteoclast marker, and the number of multinucleated TRAP positive cells were counted.

In order to test the impact of NBD-nanoparticles on pro-inflammatory responses, RAW or THP1 cells were stimulated with the periodontal pathogen *P. gingivalis*, treated with NBD-nanoparticles or empty nanoparticle and tested for expression of cytokines critical in periodontitis, such as IL-1β, IL-6, TNFα by ELISA. Differences were evaluated by ANOVA.

In vivo - NBD-nanoparticles were tested in a murine ligature-induced periodontitis model where mice received a ligation around the second molar, *P. gingivalis* infection and microinjections of NBD-nanoparticles, empty nanoparticles, or PBS in the gingiva. Gingival tissue was tested for mRNA expression of pro-inflammatory cytokines by real-time PCR. Alveolar bone loss was determined by measuring the CEJ-ABC distance. Bacterial culture from oral swabs confirmed bacterial persistence. Differences were evaluated by ANOVA.

Results: NBD-nanoparticles inhibit osteoclastogenesis directly and *P. gingivalis*-induced pro-inflammatory cytokine production. NBD-nanoparticle application inhibits the gingival expression of periodontitis-related cytokines and alveolar bone loss in a murine ligature model.

Conclusions: NBD-nanoparticle is able to inhibit osteoclastogenesis directly and pro-inflammatory cytokines production *in vitro*. This nanoparticle prevents gingival inflammation and bone loss in a murine model for periodontitis.

Keywords: NBD-nanoparticles, NF-κB pathway, periodontal disease, osteoclastogenesis, inflammation, alveolar bone loss

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CHAPTER 1: INTRODUCTION

Periodontitis, an inflammatory disease that results in a progressive loss of tooth-supporting bone, effects over 47% of the American adult population ranging from mild, moderate to severe forms of the disease (Eke et al., 2012). Destruction of tissue is pathognomonic of the disease which leads to gingival recession or periodontal pockets and the loss of alveolar bone. As a consequence of decreased tissue support around the tooth structure, mobility and eventual loss of tooth occurs.

Epidemiological studies estimate that the disease affects 70% of the US adults aged 65 years or older (Papapanou, 2012). Globally, it affects 10-15% of the adult population according to WHO (Petersen & Ogawa, 2005). Apart from being the most common chronic infection among adults, the treatment also imposes a huge economic burden globally (Loesche & Grossman, 2001). A study estimates that the projected cost could be over 1 billion dollars if only 10% of the periodontitis patients undergo periodontal therapy (Loesche & Grossman, 2001). Ironically, a majority of the people suffering from the disease belong to low-income groups and are unlikely to get the treatment done (Borrell & Crawford, 2012).

The interaction between the plaque and the host is considered the primary etiological factor in the disease. The dental plaque contains a complex of multi-bacterial species that together form a biofilm. Plaque if not removed mechanically on a regular basis, advances into a matured plaque, which consists of pathogenic

bacteria leading to dysbiotic changes. Dysbiosis, a state of impaired microbiota, is increasingly being acknowledged as a causative factor in many inflammatory diseases. This altered host-microbe cross-talk is also implicated in Periodontitis, the most common inflammatory disease of the oral cavity (Hajishengallis & Lamont, 2014).

The periodontal disease, which is one of the main causes of tooth loss, was once thought to be a localized infection (Ahn et al., 2012; Southerland et al., 2005). However, it is now considered as a risk factor for some cardiovascular, respiratory, endocrine, musculoskeletal, and reproductive system related abnormalities by being a continuous source of infection (Arigbede et al., 2012; Yakob et al., 2012).

Periodontal disease and oral pathogens:

The oral cavity, which has a diverse ecosystem, is inhabited by a plethora of bacteria which is estimated to be around 700 species. However, not all the microorganisms are harmful as the disease is caused only by an increase in the abundance of specific microbes (Suzuki et al., 2013). Among the bacteria, *Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Tannerella forsythia (*formerly *Bacteroides forsythus), Porphyromonas gingivalis, Prevotella intermedia, Treponema denticola* are important periodontal pathogens (Aruni et al., 2015)

To explain the etiology of the biofilm-induced disease, many theories have been put forth based on the role of microorganisms in the initiation and progression of the disease. Specific microorganisms, non-specific plaque, specific plaque, red

complex, key stone pathogen, poly microbial synergy and dysbiosis are a few among them (Hajishengallis & Lamont, 2012). According to the red complex concept, a group of three species is associated with the severe forms of disease including *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* (Socransky et al., 1998). Keystone pathogen theory postulates that the low abundance keystone microorganisms can disrupt the tissue homeostasis thereby causing tissue destruction (Hajishengallis et al., 2011). Porphyromonas gingivalis, one of the red complex organisms and a keystone pathogen has gained special attention partly because it is easy to culture and genetically manipulate (Hajishengallis & Lamont, 2012).

Although the etiology of periodontal disease is attributed to pathogenic microorganisms, the mere presence of periodontal pathogens does not cause tissue destruction by itself (Graves, 2008). Instead, the microorganisms involved in the pathogenesis of the disease act by modulating the host immune response (Reynolds et al., 2015). In other words, the bone loss in periodontitis is not just because of the invasion of the organism but a consequence of the pro-inflammatory cytokines produced by the host in response to the pathogenic organism (Benedetto et al., 2013; Kato et al., 2014).

Periodontal disease and Inflammation:

Inflammation is a common physiological response to harmful stimuli such as a microbial challenge in this context. Initially, the disease starts as an inflammatory response affecting the gingiva, a condition known as gingivitis.

Removal of the harmful stimuli, the supra and subgingival plaque, at this stage leads to tissue homeostasis resulting in restoration of tissue health.

However, the unremoved plaque coupled with factors such as poor oral hygiene, smoking, and other systemic conditions results in a shift from an acute stage to a chronic inflammation. The inflammation is a consequence of host responses to the persistent microbial encounter resulting from the unremoved plaque.

Pro-inflammatory cytokines or simply inflammatory cytokines have repercussions on the periodontium as a result of tissue destruction and disease progression. On the other hand, anti-inflammatory cytokines are associated with the attenuation of disease. In general, the pro-inflammatory cytokines have a destructive role while the anti-inflammatory cytokines have a protective role (Garlet, 2010).

Pro-inflammatory cytokines such as IL1-β, IL-6, TNFα are considered to be the pro-osteoclastogenic factors and contribute to the bone loss, along with Receptor activator of nuclear factor kappa-B ligand (RANKL) (Benedetto et al., 2013; Kato et al., 2014). These cytokines destroy the periodontal tissue and result in the attachment loss of the gingiva causing either gingival recession or excessive periodontal pockets (Graves & Cochran, 2003).

Interleukins are a group of cytokines produced by leukocytes. Pro-inflammatory cytokines responsible for the pathogenesis of the disease are explained briefly here.

IL-1β:

Interleukin 1 is produced by activated mononuclear phagocytes and is produced in response to bacterial antigens and their products (Beuscher et al., 1990). There are two types of interleukin1; interluekin1α, interleukin 1β. Though the two subtypes of interleukin 1 bind to the same receptor, they have distinct functions. IL-1β is produced by blood monocytes, tissue macrophages, skin dendritic cells, and brain microglia. The precursor of IL-1β is inactive and it is activated by cleavage of caspase-1. The precursor is released in response to TLR, active complement proteins, cytokines and IL-1 itself (Garlanda et al., 2013). Studies have shown that IL-1β, found in abundance in the crevicular fluid of gingiva, is a potent inducer of bone destruction and cause tissue destruction in inflammatory diseases including periodontitis (Hou et al., 1995; Hönig et al., 1989).

IL-6:

IL-6 is a pro-inflammatory cytokine, which has been found in the gingival crevicular fluid of periodontal patients. It plays a key role in acute inflammation and induces bone destruction (Graves, 2008). It is produced by B-lymphocytes, monocytes and macrophages, keratinocytes, endothelial cells, and fibroblasts. IL-6 is released in response to a stimulus such as bacteria or its products and other cytokines such as IL-1β and TNFα (Irwin & Myrillas, 1998). The pro-inflammatory cytokine aids in

the pathogenesis of the periodontal disease by acting as a bone resorbing factor (Graves, 2008).

Cytokine	Effect
IL-1β	Bone resorption; pro-inflammatory; fever
TNF-α	Bone resorption; pro-inflammatory; fever; synergistic with IL-1β
IL-6	B-cell differentiation; antibody production; osteoclast differentiation

Table 1. Cytokines effect in inflammatory conditions

Reused from A clinical guide to periodontology: Pathology of periodontal disease Hasan & Palmer, British Dental Journal.

TNFα:

Tissue necrosis factor α is considered one of the most potent cytokines which induces osteoclastogenesis in inflammatory conditions such as rheumatoid arthritis, orthopedic implant loosening, periodontitis and other chronic inflammatory osteolytic diseases (Garlet, 2010; Lam et al., 2000; Zhang et al., 2001). TNFα has been confirmed to upregulate the other cytokines such as IL-1β and IL-6 and has been found in abundance in the gingival crevicular fluid (Benedetto et al., 2013; Garlet, 2010). TNFα acts in the progression of the periodontal disease by multiple mechanisms such as expression of chemokines, inflammatory mediators, matrix metalloproteinases, osteoclastogenesis, and apoptosis of matrix-producing cells (Graves & Cochran, 2003).

In summary, these pro-inflammatory cytokines break down the tissue causing attachment loss and bone resorption.

Periodontal disease and bone loss:

Alveolar bone loss in periodontitis is the main factor including attachment loss, which is responsible for tooth mobility or even loss of the tooth. Bone loss is caused by the osteoclasts by bone resorption. On the other hand, osteoblasts are the cells that help in the bone deposition. In fact, the action of both osteoblasts and osteoclasts is necessary for normal development, remodeling and functioning of an organism. In health, the osteoblastic and osteoclastic activity are in a balanced state where the amount of bone resorption equals the amount of deposition resulting in a healthy maintenance of bone (Hienz et al., 2015).

Osteoclastogenesis is the development of the bone resorbing cells, the Osteoclasts. Osteoclasts are terminally differentiated large cells with multiple nuclei and originate from mononuclear cells of the hematopoietic stem cell lineage (Florencio-Silva et al., 2015). Their unique structure consists of a ruffled border seen on the cell surface that helps in secretion of enzymes as well as uptake of matrix components. A Clear zone is seen encircling the ruffled border and helps in resorption (Teti et al., 1991). Vacuoles and vesicles help in secretion of enzymes, digestion or transport of products within the cell (Holtrop & King, 1977).

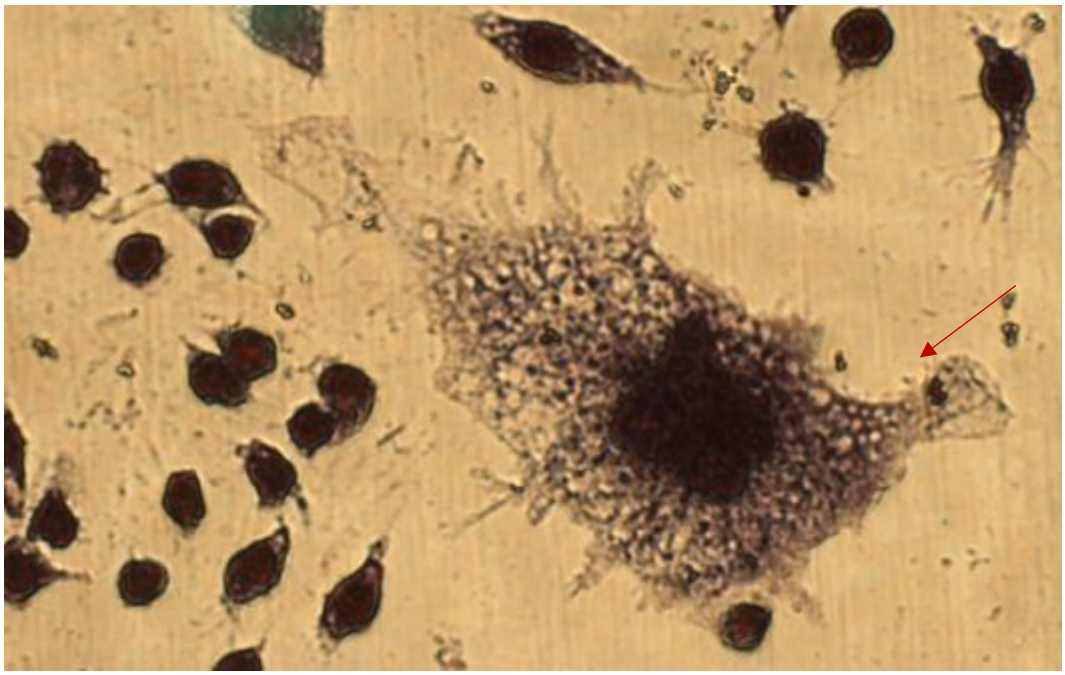

Figure 1: Tartrate resistant acid phosphatase positive (TRAP) Osteoclast
Arrow indicates the TRAP-positive cell: osteoclast.

The lysosomes contain large amounts of Tartrate-resistant acid phosphatase (TRAP) and is a useful cytochemical marker (Ballanti et al., 1997). Excessive osteoclastogenesis shifts the balance in favor of extensive bone loss as seen in periodontitis.

NFκB Signaling pathway and Periodontal disease:

NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) signaling pathway is critical in osteoclastogenesis and infection-induced pro-inflammatory responses for being at the juncture of diverse signaling pathways (Abu-Amer, 2013; Lawrence, 2009). Blocking this pathway has become an attractive target in

inflammatory diseases such as cancer and chronic inflammatory diseases and is possible with Nemo Binding Domain (NBD) inhibitory peptide (Pan et al., 2011). NF-κB consists of transcription factors which are pivotal in cellular growth and development, regulating immune and inflammatory responses, and apoptosis (Oeckinghaus & Ghosh, 2009). These factors are responsible for a number of disease states such as atherosclerosis, heart disease, rheumatoid arthritis, cancer and also Periodontitis (Nichols et al., 2001; Okamoto, 2006; Ross et al., 2001; Valen et al., 2001; Gilmore et al., 2002).

The NF-κB protein family is divided into two subfamilies: the ‘’NF-κB’’ proteins and ‘’Rel’’ proteins. NF-κB proteins include p105, p100. The Rel subfamily includes c-Rel, RelB, RelA (aka p65). Rel proteins contain C-terminal transactivation domains and can activate transcription. Long C- terminal domains with multiple copies of Ankyrin repeats on the NF-κB subfamily of proteins make them inactive. These proteins become short to get active by either limited proteolysis or arrested translation (p105 to p50 and p100 to p52). This necessitates NF-κB subfamily to form dimers with members of the Rel subfamily to activate transcription (Gilmore, 2006). The active form of NF-κB is thus a heterodimer consisting p65 and p50 subunits (Barnes & Karin, 1997).

As the NF-κB pathway is involved in many important cellular responses, its tight regulation is justified. This is done by the interaction with the inhibitory proteins IkB such as IkBα, IkB β, IkB γ, IkB ē which have different affinities for individual

NF-κB dimers. As such, in most of the cells, NF-κB is present in an inactive form in the cytoplasm along with IkB protein (Oeckinghaus & Ghosh, 2009). The NF-κB signaling is activated by a stimulus, bacteria/bacterial products in this circumstance. The activation of NF-κB requires IKKα, IKKβ and the regulatory protein NF-κB essential modifier IKK γ (or NEMO) which together constitute the IKK complex (Hacker & Karin, 2006).

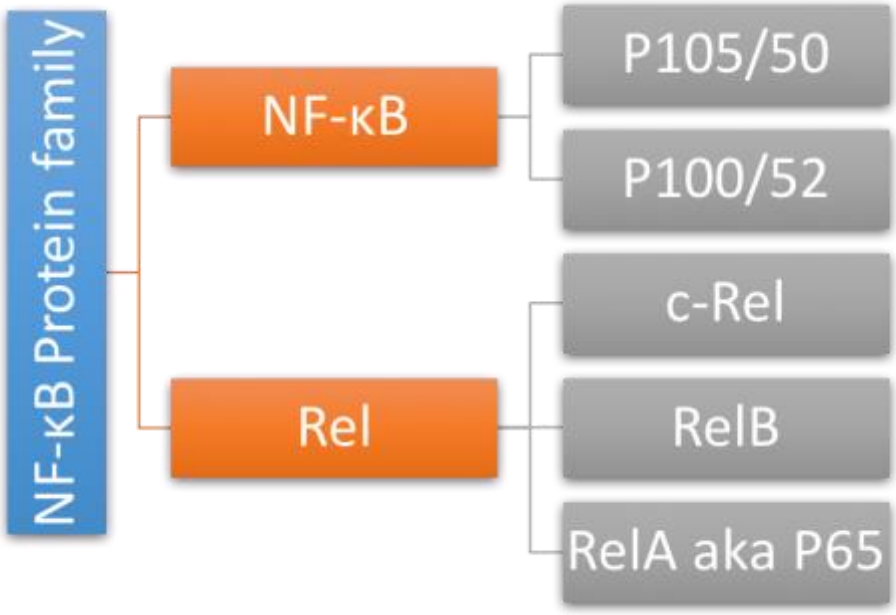

Figure 2: The NF-κB Protein family

Image showing the classification of mammalian NF-κB proteins.

The IKK complex phosphorylates IKBα leading to ubiquitination and degradation. NF-κB which is now free can translocate into the nucleus where it initiates transcription (Oeckinghaus & Ghosh, 2009).

NF-κB activation can occur by two main pathways: Canonical or classical and non-canonical pathway. Recent studies show that the canonical and the non-canonical

pathway are involved in innate immunity and adaptive immunity respectively (Bonizzi & Karin, 2004).

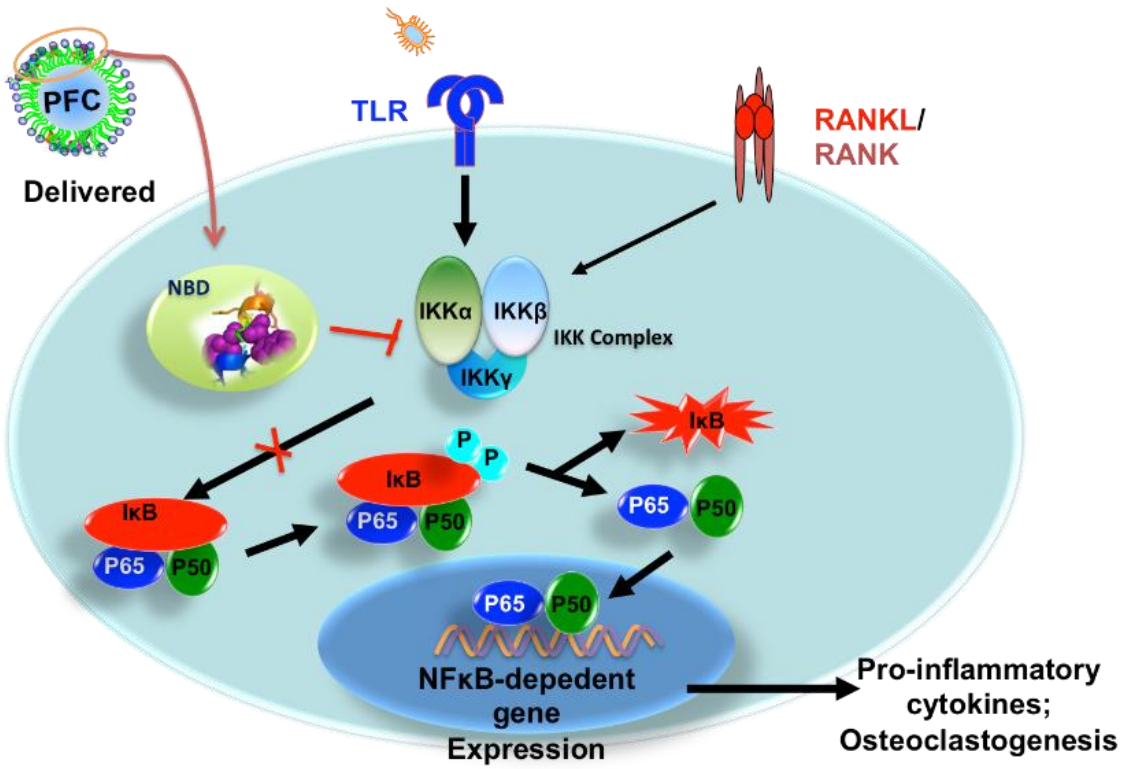

Figure 3: Schematic depiction of the NF-κB pathway

The image depicts the mechanism of pro-inflammatory cytokine production and osteoclastogenesis through activation of NF-κB.

The classical pathway can be stimulated by cytokines such as TNFα, interleukin 1 (IL-1), antigens and toll-like receptors (TLR4). It is mainly concerned with the phosphorylation of IkB α and is dependent on IKKβ and IKKγ of the IKK complex, resulting in the translocation of P65 protein heterodimers (Hayden & Ghosh, 2004; Oeckinghaus et al., 2011).

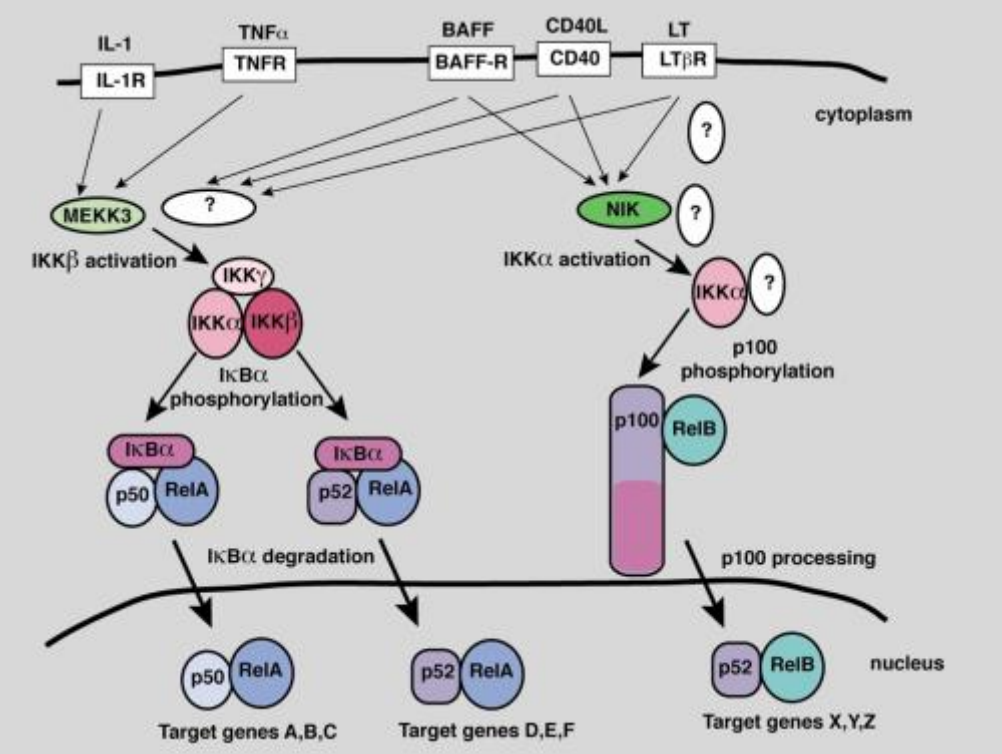

Figure 4: Canonical (Left) and Non-canonical (Right) Pathways for the Activation of NF-κB

Figure reused with permission from Elsevier, Molecular cell, Two Pathways to NF-κB, Pomerantz, Baltimore, October 2002.

On the other hand, the non-canonical pathway is induced by specific TNF cytokines such as CD40 ligand, BAFF, and lymphotoxin-β except TNFα (Bonizzi & Karin, 2004; Oeckinghaus et al., 2011). It is dependent on IKKα-mediated phosphorylation of P100- RelB, resulting in the formation of p52-RelB complexes (Hayden & Ghosh, 2004).

The NF-κB pathway is important in pro-inflammatory signaling through the production of cytokines, chemokines, and adhesion molecules (Lawrence, 2009). In addition, NF-κB signaling transduction is pivotal in the downstream of

RANK/RANKL pathway that leads to the development of osteoclasts (Yamaguchi et al., 2012).

Periodontal pathogens, Porphyromonas gingivalis, and NFκB Signaling pathway:

Bacteria, bacterial products, and viruses are known to be the inducers of NF-κB, which controls of transcription of around 150 target genes (Pahl, 1999). Among the bacteria, *Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Tannerella forsythia (*formerly *Bacteroides forsythus), Porphyromonas gingivalis, Prevotella intermedia, Treponema denticola* are important periodontal pathogens and are known to be inducers of NF-κB (Kobayashi-Sakamoto et al., 2004; Walter et al., 2004; Hasebe et al., 2004; Huang et al., 2004; Kim et al., 2004; Tanabe et al., 2008; Tiranathanagul et al., 2004). Also, bacterial lipopolysaccharide (LPS) has been shown to activate NF-κB signaling pathway (Sen & Baltimore, 1986).

Porphyromonas gingivalis is one of the major etiological organisms in the pathogenesis of the periodontal disease that is also known to be an inducer of the NF-κB (Groeger et al., 2017; Lamont & Jenkinson, 1998). Studies have shown that LPS, one of the major constituent of the outer membrane of *Porphyromonas gingivalis*, induces NF-κB (Ding et al., 2013). Also, studies have found that the activation of NF-kB by *P. gingivalis* is through IkB kinase (IKK) complex (Carayol et al., 2006). In addition, IKK γ, the regulatory protein is essential for the activation of IKK complex in inflammation (May et al., 2000).

LPS consists of three parts: lipid A, a core oligosaccharide, and an O side chain (Raetz & Whitfield, 2002). Pathogen-associated molecular patterns (PAMPs) are structures within a class of microbes that are recognized by pattern recognition receptors (PRRs) present on the cells of innate immunity system (Mogensen, 2009). Lipid A of LPS is the main PAMP that is recognized by toll-like receptors, a pattern recognition receptor (PRR). The LPS binds to toll-like receptors, mainly TLR4 and upregulates the expression of nuclear factor-κB (NF-κB) (Wang et al., 2000). Activation of NF-κB signaling pathway by Porphyromonas gingivalis leads to the production of pro-inflammatory cytokines particularly interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and IL-6 and osteoclastogenesis (Choi et al., 2005) (Diya et al., 2008).

RANKL/RANK/OPG Pathway, NF-κB, and Osteoclastogenesis:

Many factors such as macrophage colony- stimulating factor (M-CSF), Receptor activator of nuclear factor kappa-B ligand (RANKL) influence the formation of osteoclasts the RANKL/RANK/OPG Pathway which is upstream of the NF-κB signaling cascade.

NF-κB pathway is downstream of RANKL/RANK pathway and it is now established that this pathway is critical and essential for osteoclastogenesis (Boyce, 2013). RANKL, a member of TNF family, is a homotrimeric transmembrane protein secreted by osteoblasts and stromal cells (Nelson et al., 2012). It is expressed in bone and bone marrow, lymph nodes, thymus, spleen, mammary glands and the brain (Liu & Zhang, 2015).

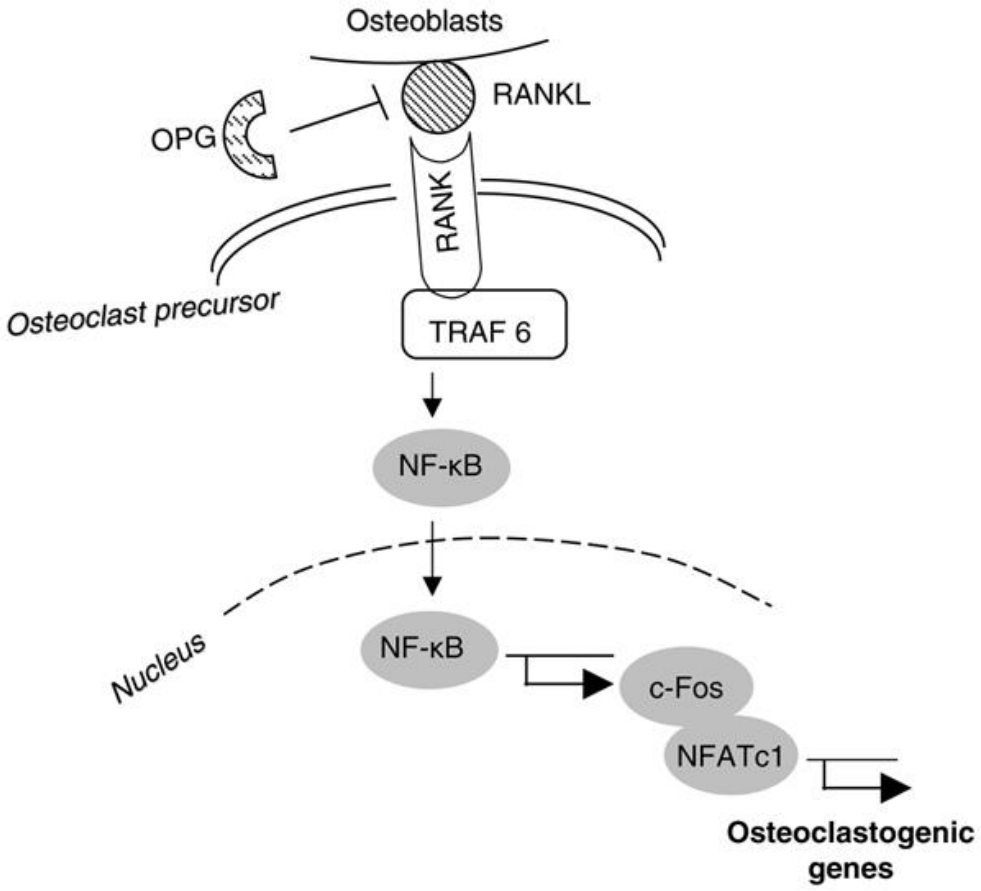

Figure 5: The RANKL/RANK/OPG pathway for normal osteoclastogenesis.
Picture reused with permission from Dr. Brendan F Boyce, Biology of RANK, RANKL, and Osteoprotegerin, Arthritis Research & Therapy2007

In Rheumatoid arthritis, RANKL is highly expressed and appears to be responsible for the destruction of tissue at the joint (Geusens, 2012). Binding of RANKL to its receptor RANK on osteoclast precursors leads to the formation of osteoclasts (Khosla, 2001). To counteract the production of osteoclasts, a factor called osteoprotegerin (OPG) is produced which binds to RANKL, thus acting as a decoy receptor and inhibiting the osteoclastogenesis.

Receptor activator of NF-κB (RANK) is a member of tumor necrosis factor receptor (TNFR) family and a type 1 homotrimeric transmembrane protein present in normal

cells, mammary glands and in some cancer cells (Boyce & Xing, 2007). RANKL binds to its receptor RANK resulting in osteoclastogenesis via activation of NF-κB. RANKL/RANK/OPG axis is hence considered the central controller of the osteoclastogenesis and function (Florencio-Silva et al., 2015; Kobayashi, Udagawa, & Takahashi, 2009).

NF-κB pathway and Pro-inflammatory cytokines:

A coordinate regulation of the signaling pathways that regulate the expression of pro-inflammatory and anti-inflammatory cytokines is necessary to maintain health. This is because excessive production of pro-inflammatory cytokines drives the tissue in favor of destruction. The significance of NF-κB pathway in the expression of pro-inflammatory genes has been well-documented (Lawrence, 2009). These pro-inflammatory genes are responsible for the expression of cytokines, chemokines, and adhesion molecules. Although cytokines produced act independently, there is an associated crosstalk with other cytokines. NFκB signaling pathway exhibits a broad control over the network and cascade of cytokines by regulating the genes involved in the production of cytokines.

As previously mentioned, current evidence suggests that the pro-inflammatory cytokines such as IL1-β, IL-6, TNFα contribute to the tissue destruction and bone loss in periodontal disease (Benedetto et al., 2013; Kato et al., 2014; Graves & Cochran, 2003). Furthermore, the periodontal pathogens *Actinomycetes actinomycetemcomitans* and *P. gingivalis* have been shown to induce the production of IL-1β, TNF-α, IL-6 through NFκB pathway (Kayal, 2013). NF-κB

pathway is critical in the expression of cytokine genes involved in the pathogenesis of periodontitis: IL-1β , IL-6, and TNFα (Cogswell et al., 1994) (Libermann & Baltimore, 1990; Liu et al., 2000). Hence, the NF-κB pathway which includes both the canonical and the non-canonical pathway can be described as the “mastermind” in the regulation of these pro-inflammatory cytokines which are critical in the pathogenesis of periodontal disease.

NBD-nanoparticles:

Many authors have described this labyrinthine pathway as a “holy grail” of targeting the pathway for therapeutic purposes (Firestein, 2004; Lawrence, 2009). One of the approaches is to block the pathway with the help of an NBD peptide. The NEMO binding domain (NBD) peptide is shown to effectively block the association of NEMO with IKK complex thereby inhibiting NF-κB activation. This peptide targets only the inflammation induced NF-κB activity, while sparing the protective functions of basal NF-κB activity displaying fewer undesired side effects (Strickland & Ghosh, 2006). Also, the traditional lipid bilayer system is ineffective in drug delivery as the peptides are subjected to rapid protease degradation and limited bioavailability due to their inability to pass through the cell membrane. The peptide is incorporated into the outer lipid monolayer of the stable perfluorocarbon nanoparticle system for effective drug delivery that ensures inhibition of NF-κB but not complete suppression (Pan et al., 2011b).

The use of nanoparticles increases stability, loading capacity, and facilitates transmembrane transportation of the drug. The efficacy of the NBD-nanoparticles

in inhibiting bone loss in relation to periodontitis has not been tested to date. The current treatment procedures for periodontitis are expensive, unaffordable by those who require the treatment the most and require long-term maintenance by the patients to be effective. The NBD-nanoparticles may prove to be a novel therapeutic drug treating the disease more effectively, thus reducing the severity of the disease.

HYPOTHESIS

We hypothesized that the NBD-nanoparticles can lead directly to inhibition of osteoclastogenesis and pro-inflammatory cytokines production in vitro. We also hypothesized that this nanoparticle inhibits gingival inflammation and prevents bone loss in a murine model for periodontitis.

CHAPTER 2: MATERIALS AND METHODS

***In vitro* experiments:**

Osteoclastogenesis:

RAW 264.7 cells (ATCC CLR-2278) were cultured (10^3 cells) in a 96 well plate in α-MEM supplemented with 10% FBS and 100ng/ml murine soluble RANKL (sRANKL) (Peprotech®). Nanoparticles of different dosages were added into the cell culture (120, 600, 1200 and 2400 pM). Five days later, the cells were stained for the enzyme tartrate-resistant acid phosphatase (TRAP) using the Leukocyte Acid Phosphatase TRAP Kit (Sigma-Aldrich®). The number of multinucleated TRAP-positive cells were counted which represent the multinucleated osteoclasts. (Jacome-Galarza et al., 2013). NBD-nanoparticles and nanoparticles were obtained from Washington University at St. Louis in collaboration with the laboratories of Dr. Samuel A. Wickline and Dr. Hua Pan.

Pro-inflammatory cytokines expression:

The RAW 264.7 cells (ATCC CLR-2278) and THP 1 cells (Invivogen) were separately stimulated with *P. gingivalis* 33277 (ATCC®) at MOI of 10:1 overnight. Cells were treated with different dosages of nanoparticles (120, 600, 1200 and 2400 pM) along with *P. gingivalis* 33277 (ATCC®). The culture supernatant was harvested after incubating overnight. The cytokine expression of IL-1β, IL-6, and TNFα was measured by enzyme linked immunosorbent assay (ELISA).

***In vivo* Experiments:**

Murine ligature induced periodontitis model:

Mice received a ligature around the second molar, placed in the gingival sulcus. (Abe & Hajishengallis, 2013). The ligature-induced periodontitis model is a preferred method as it drastically reduces the time to develop periodontitis and by exaggerating inflammation and bone loss (Molon et al., 2014; Oz & Puleo, 2011).

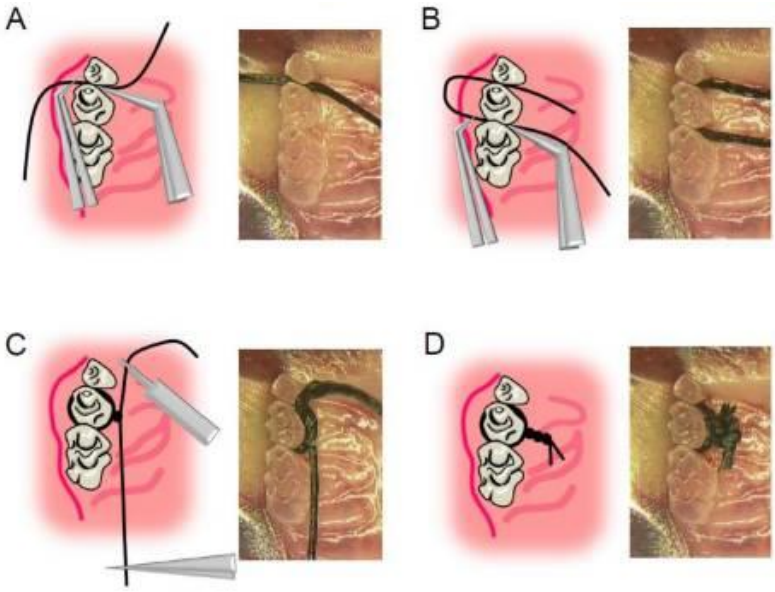

Figure 6: illustration of Steps involved in the ligation procedure

Picture reused with permission from Journal of Immunological Methods, Abe & Hajishengallis, (2013). Optimization of the ligature-induced periodontitis model in mice.

A) 5–0 silk suture was passed in between the second molar and third molar using Dumont forceps. (B) Suture was passed through between first molar and second molar using Dumont forceps. (C) Suture was looped around the second molar (taking care to remove the slack) using suture-tying forceps. (D) Suture was tied firmly using a triple-knot and excess suture was cut using spring scissors.

The mice were infected with 1 x 10^9 colony-forming units of *P. gingivalis* (3327; American Type Culture) or 2% carboxymethylcellulose vehicle as a control every other day for 3 times. Immediately after the ligation, micro-injections of 10µl of 1.2 nM NBD-Nanoparticles, nanoparticles or phosphate buffer solution (PBS) was given in the palatal gingiva between first and second molars on day 1.

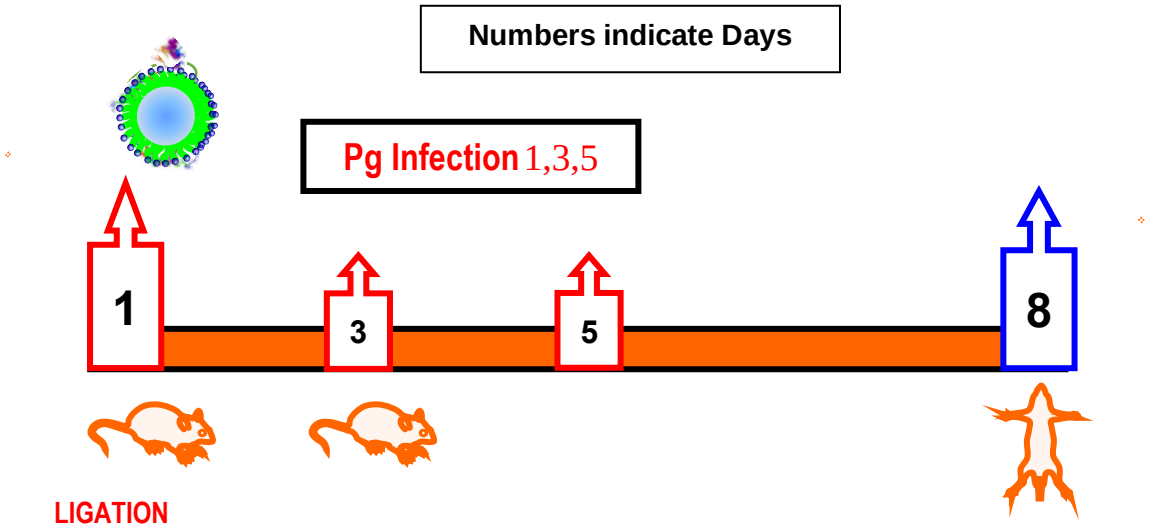

Figure 7. Schematic depiction of the protocol for periodontitis induction *in vivo***.**

On the first, third and fifth day of ligation, microinjections of NBD- nanoparticles, nanoparticles or phosphate buffer solution (PBS) was given in the palatal gingiva between first and second molars. Additionally, they were infected with *P. gingivalis* on days 1, 3, and 5. Seven days later the mice were harvested. Numbers indicate Days.

Gingiva harvest and quantification of cytokines:

After seven days of ligation, the mice were sacrificed and the gingival tissue was harvested.

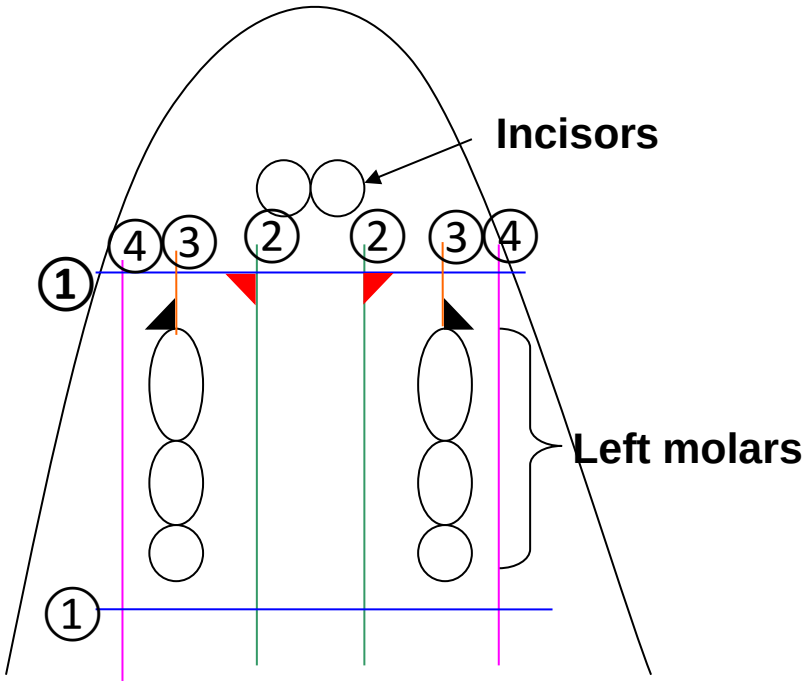

Figure 8: Illustration of the steps involved in the harvest of gingiva.
Incision ①, ②, ③ were made first. The red triangle part of the gingiva was grasped and the lingual part of gingiva was pulled. Then, incision ④ was made and the black part of the gingiva grasped and the buccal part of the gingiva pulled.

RNA was isolated using RNeasy kit (Qiagen). RNA was reversed-transcribed and mRNA expression of cytokines - IL-1β, IL-6, TNFα, and RANKL was determined by qualitative real-time PCR.

Jaw harvest and Morphometrical analysis:

The skulls were subjected to boiling at 15psi for 10 minutes. After defleshing, the maxillae were separated from the skulls. Maxillae were cleaned with gentle brushing followed by bleaching overnight. Later, they were stained with 0.5% eosin and 1% methylene blue (Abe & Hajishengallis, 2013). Alveolar bone heights were assessed using a 40 X objective under a Nikon SMZ800 microscope (Nikon Instruments Inc., NY, USA).

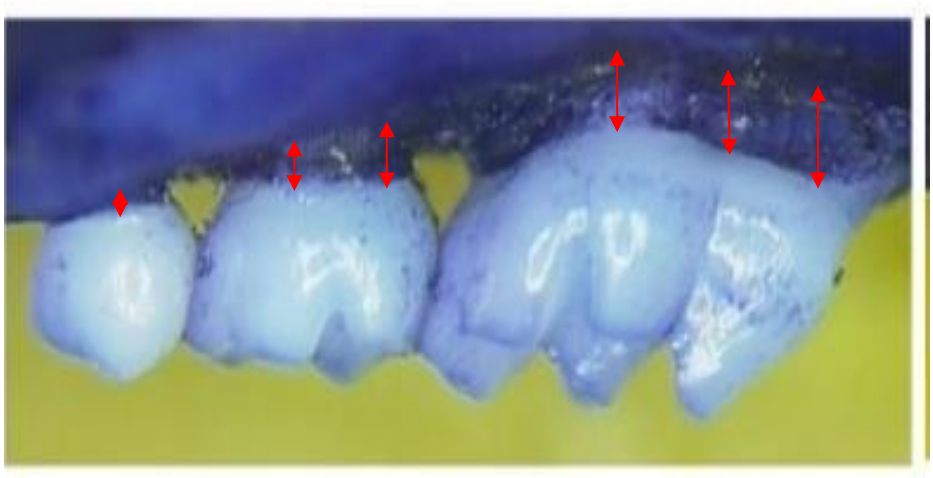

Figure 9: Picture of mice maxillary teeth showing the sites of measurement.

Arrows indicate the site of measurement. Measurements were taken at six different sites- the mesial cusp, buccal groove and distal cusp of the first molar, the mesial cusp and distal cusp of the 2nd molar, and buccal cusp of the third molar.

Bone heights were measured morphometrically and the images of the maxillae were captured using a Nikon Digital Sight DS-U3 camera controller (Nikon Instruments Inc.). Measurements were taken at six different sites- the mesial cusp, buccal groove and distal cusp of the first molar, the mesial cusp and distal cusp of

the 2nd molar, and buccal cusp of the third molar. The readings of CEJ-ABC distance at each site were totaled for each mouse. Bone loss was calculated by subtracting the six-site total CEJ-ABC distance for the ligated mice from the six-site total CEJ-ABC distance of the unligated mice. Micro-CT was performed in the Department of Radiology at University of Louisville.

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.

Vertebrate Animals: C57BL6 mice 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, IACUC number: 15323.

CHAPTER 3: RESULTS

The overall strategy of this research is to investigate the ability of NBD-nanoparticles to directly inhibit osteoclastogenesis, and the pro-inflammatory cytokine response to a bacterial stimulus using *in vitro* studies. Also, to investigate the therapeutic ability of NBD-nanoparticles to inhibit pro-inflammatory cytokine production in the gingiva and reduce alveolar bone loss in a murine ligature induced periodontitis model.

1) NBD-nanoparticle inhibits RANKL-induced osteoclastogenesis:

Bone loss in periodontitis is a result of an imbalance in the osteoblastic and osteoclastic activity. Since the current evidence attributes the bone loss to osteoclastogenesis through the NF-κB pathway that is downstream of RANKL/RANK pathway, we first investigated whether the NBD-nanoparticle can prevent the RANKL-induced osteoclastogenesis *in vitro*.

Since osteoclasts are derived from monocyte precursors, we used the RAW cell line cells which are murine monocyte/macrophages. Furthermore, RAW cells are the most extensively used cell line for decades to study the differentiation of osteoclasts (Collin-Osdoby et al., 2003; Jacome-Galarza et al., 2013).

In order to test the ability of NBD-nanoparticles to prevent RANKL-induced osteoclastogenesis, we examined the cells through microscopy, and osteoclastic cell formation was determined by the number of multinucleated TRAP positive cells. Cells with three or more nuclei were considered to be osteoclasts. Controls included treatment with empty nanoparticles and no treatment. Cells with no stimulation (RANKL) were also used.

In the cells without RANKL-stimulation and with or without NBD nanoparticle treatment, there were virtually no TRAP-positive cells indicating that RANKL is required for differentiation into osteoclasts. As expected, the cells incubated with RANKL stimulated the formation of a large number of TRAP positive cells. This indicates a crucial role of RANKL in initiating the RANKL/RANK pathway towards osteoclastogenesis. After we treated the RAW cells with NBD-nanoparticles, the formation of TRAP positive cells was significantly reduced (Fig 10, 11), while the empty nanoparticles without NBD did not inhibit osteoclastogenesis (Fig 10). We further counted the number of osteoclasts in each well. Our results showed that NBD- nanoparticle effectively inhibited the osteoclastogenesis.

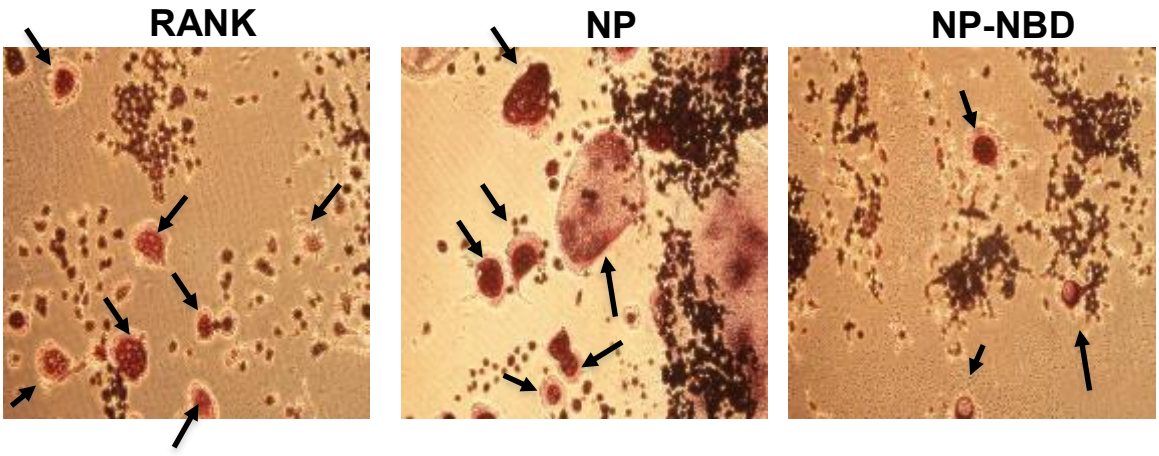

Figure 10. NBD-nanoparticle inhibits RANKL-induced osteoclastogenesis: RAW 264.7 cells were treated with RANKL and 2.4µM of NBD-nanoparticles (NBD-NP) or Nanoparticles (NP). Untreated cells (RANKL) or empty Nanoparticle (NP) treated cells were used as controls. The cells were cultured for 5 days and stained with TRAP.

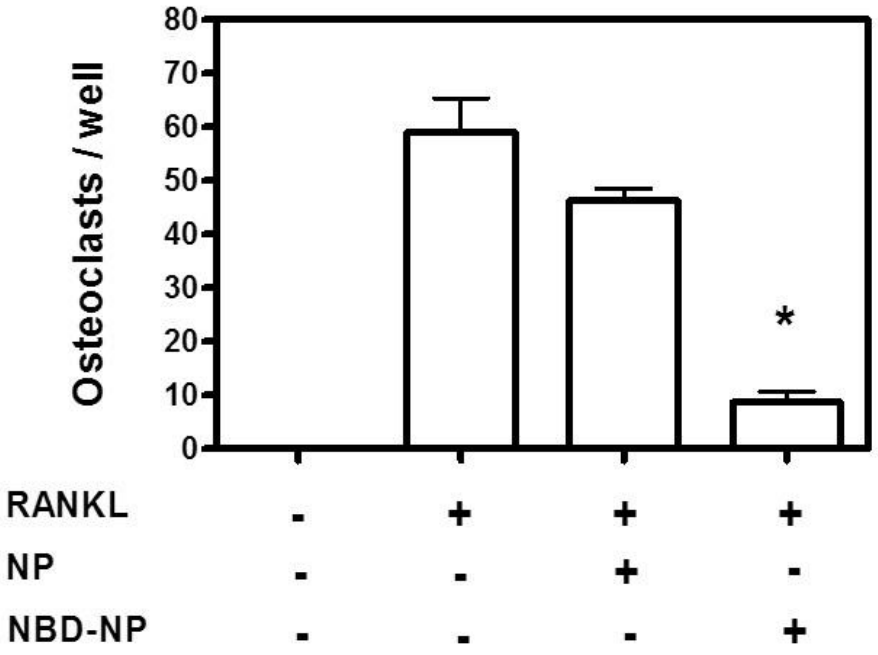

Figure 11. NBD-nanoparticle inhibits RANKL-induced osteoclastogenesis. RAW 264.7 cells were treated with RANKL and 2.4μM of NBD-nanoparticles (NBD-NP). Untreated (RANKL only) and empty nanoparticle treated cells were used as controls. The cells were cultured for 5 days and stained with TRAP. The multinucleated TRAP positive cells were counted per well. * $p<0.05$

2. NBD-nanoparticle inhibits *P. gingivalis*-induced pro-inflammatory cytokine production:

It has already been reported that the NF-κB pathway is critical in the infection-induced pro-inflammatory cytokine production. Moreover, considering the role of inflammatory cytokines in the destruction of tooth supporting tissue in periodontitis, we wanted to examine if NBD-nanoparticles will inhibit infection- induced pro-inflammatory cytokine expression relevant to periodontitis- IL-1β, IL-6 and TNFα.

For measuring the expression of IL-1β, IL-6 and TNFα by ELISA, either RAW cells or THP1 cells were incubated overnight and the supernatant was harvested. The cells that did not stimulated with *P. gingivalis* showed nearly negligible amounts of IL-1β, suggesting that there was no NF-κB activation in the absence of infection. In contrast, a dose dependent significant decrease in the amount of IL-1β was noted in the cells that received NBD-nanoparticle treatment. At a treatment dose of 2.4 μM of NBD nanoparticles, the amount of IL-1β was almost equivalent to the unstimulated cells. This data confirms that the NBD-nanoparticles effectively block the production of IL-1β (Fig.12).

Furthermore, cells expressed IL-6 responding to *P. gingivalis* stimulation, while the amount of IL-6 was significantly reduced in the NBD-nanoparticle-treated human monocyte cells. A dose-dependent decrease was observed from 0.6 to 1.2 μM NBD-nanoparticles (Fig.12). Similarly, in murine macrophage cells, a small amount of IL-6 expression was observed in the unstimulated cells treated with the empty

nanoparticle (Fig. 13). The amount of IL-6 after treatment with NBD-nanoparticles decreased to the level of unstimulated cells confirming that the NBD-nanoparticle is capable of inhibiting the IL-6 production *in vitro* (Fig. 13).

Next, we investigated the expression of TNFα in murine macrophage cell line for testing the efficacy of NBD-nanoparticles. Data showed that the unstimulated cells only produce tiny amount of TNFα. Not surprisingly, *P. gingivalis* stimulation induced TNFα production. Our data indicate a dosage-dependent decrease in the TNFα levels after treatment with NBD-nanoparticles in *P. gingivalis*-infected cells (Fig.13). This suggests that the NBD-nanoparticle is effective in suppressing TNFα production.

In summary, our results corroborate that the NBD-nanoparticles are able to inhibit the infection-induced production of IL-1β, IL-6 and TNFα, which are important cytokines in the pathogenesis of the periodontal disease.

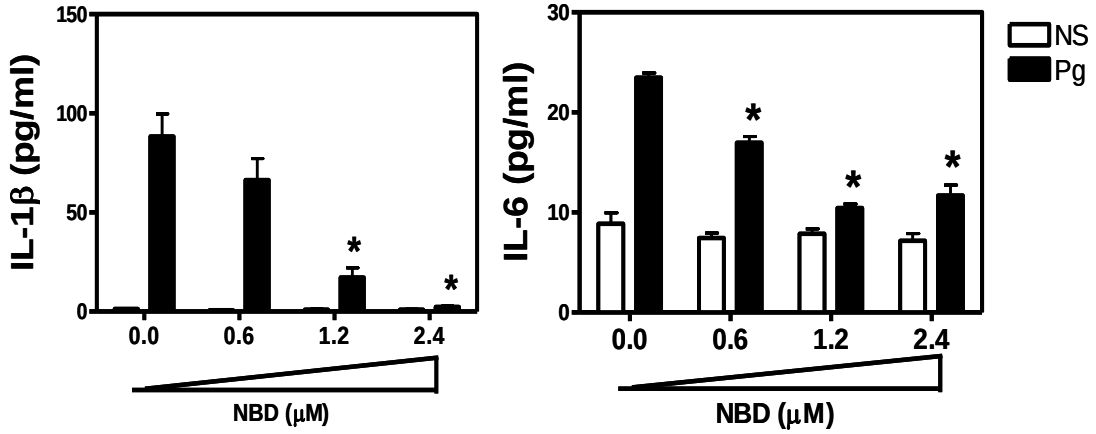

Figure 12. NBD-nanoparticle inhibits *P. gingivalis*-induced pro-inflammatory cytokine production by human monocytes: THP1 cells were treated with different dosages (0.6, 1.2, and 2.4μM) of NBD-nanoparticles (NBD) at the time of *P. gingivalis* (Pg) infection (MOI 10:1). The cells without stimulation (NS) were used as controls. The cells were cultured overnight and supernatant were tested for production of (A) IL-1β and (B) IL-6. Data presented as mean ± SD (n = 3).

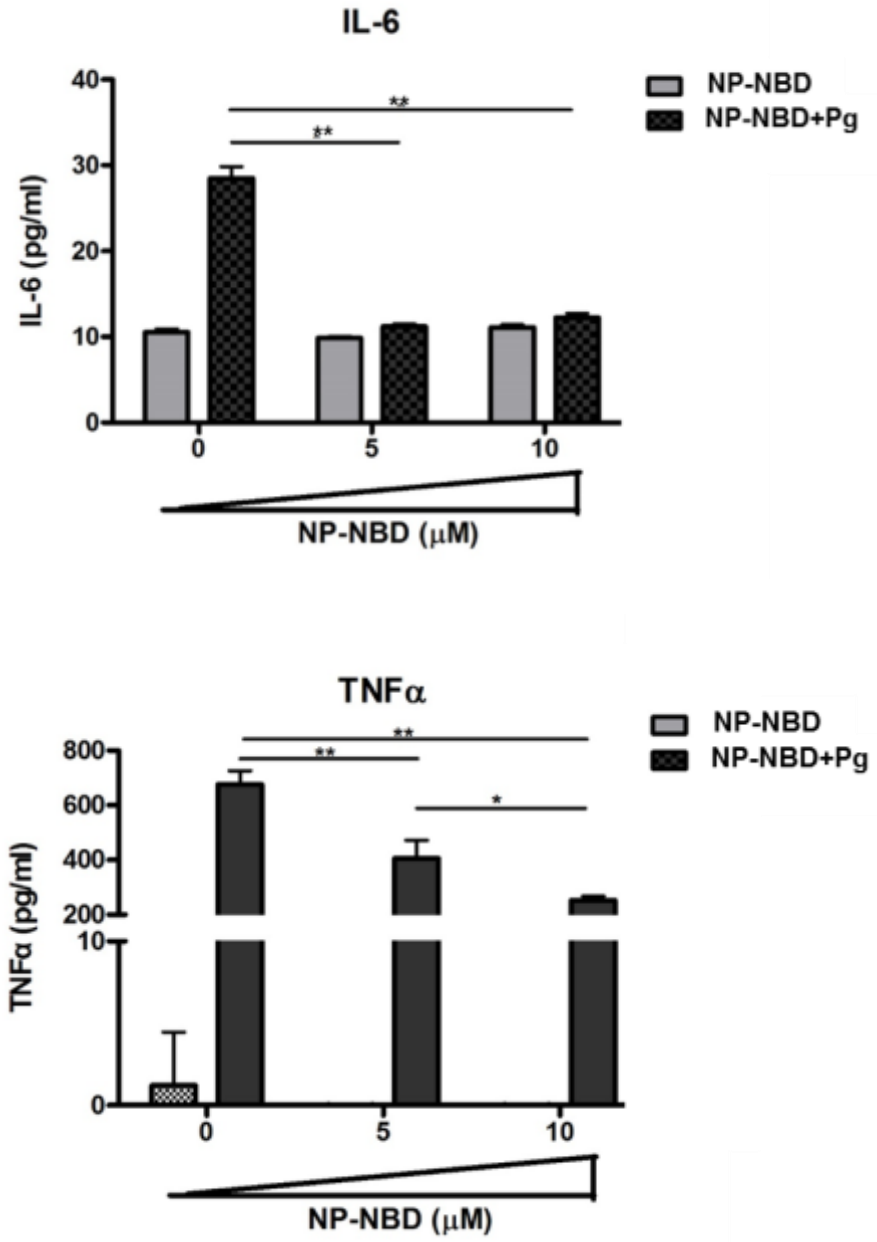

Fig. 13. NBD-nanoparticle inhibits *P. gingivalis*-induced pro-inflammatory cytokine production by RAW cells. RAW cells were treated with different dosages of NBD-nanoparticles (NBD) at the time of *P. gingivalis* (Pg) infection (MOI 10:1). The cells were cultured overnight and supernatant were tested for production of (A) TNFα and (B) IL-6. * $p<0.05$, ** : $p<0.01$

***In vivo* experiments:**

The overall strategy of the *in vivo* experiments is to determine the ability of NBD-nanoparticles to effectively control the inflammation and inhibit alveolar bone loss. For this purpose, a murine ligature-induced Periodontitis model was used (Abe & Hajishengallis, 2013).

3. NBD-nanoparticle treatment inhibits expression of periodontitis-related cytokines:

We have established that the NBD-nanoparticle can inhibit inflammatory cytokine production *in vitro*. In addition, literature provides evidence that the production of pro-inflammatory cytokines via NF-κB activation as a host response to the microbial attack leads to tissue destruction in periodontitis. So, we wanted to investigate the NBD-nanoparticle's ability to inhibit inflammation *in vivo* using a murine ligature-induced periodontitis model.

C57/BL6 mice were ligated and received micro-injections of NBD-nanoparticles. Additionally, the ligated mice were infected with *P. gingivalis* three times every other day. Controls included

ligated mice treated with empty nanoparticles or no treatment. On day 8, the mice gingivae were harvested and tested for expression of cytokines, including RANKL, IL-1β, IL-6 and IL-17 by quantitative RT-PCR.

Our results demonstrated a high level of expression of RANKL, IL-1β and IL-6 except IL-17 in the untreated mice (Fig. 14). Furthermore, the empty nanoparticle

treated mice had no significant difference in the expression levels from the untreated mice, although IL-17 relative levels were increased after treatment with empty nanoparticles. Nonetheless, literature suggests a controversial dual role of IL-17 in protection and progression of inflammatory diseases (Miossec et al., 2009; Yu et al., 2007). However, the NBD-nanoparticle seemed to have a consistent suppressive effect on all the tested cytokines, which is indicated by the decreased relative levels of expression.

These data show that NF-κB inhibition by the NBD nanoparticle results in a decreased expression of cytokines – RANKL, IL-1β, IL-17 and IL-6 in the gingiva. In addition, these results indicate that the NBD-nanoparticle is able to inhibit inflammation in the gingival tissues in the murine periodontitis model.

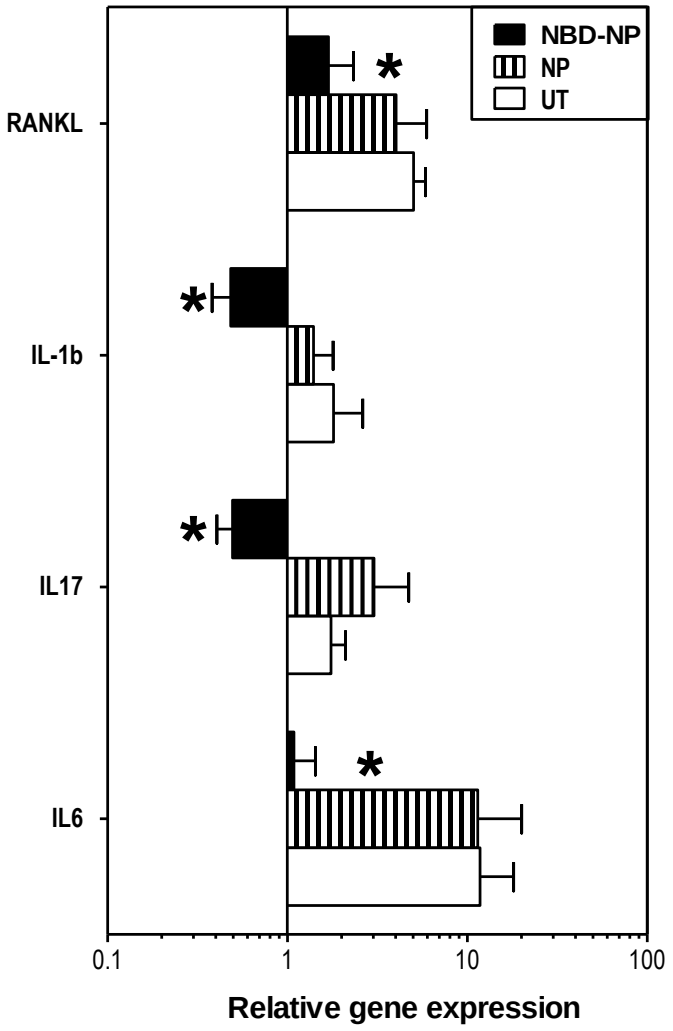

Figure 14. NBD-nanoparticle treatment inhibits expression of periodontitis-related cytokines. Relative expression of pro-inflammatory cytokines in the gingivae of mice treated with NBD-nanoparticle (NBD-NP), empty nanoparticles (NP), or without treatment (UT). Quantitative real-time PCR (qPCR) was used to determine gingival mRNA expression levels for the indicated molecules (normalized against GAPDH mRNA levels). The gingivae used were excised from

C57/BL6 mice. Results are shown as fold change relative to sham-ligated mice, respectively. Each data point represents the mean ± SD of 5 separate expression values, each value corresponding to qPCR analysis of an individual mouse. Asterisks indicate statistically significant ($p < 0.05$) differences between NBD-nanoparticle treated mice and other groups.

4. NBD-nanoparticle inhibits alveolar bone loss in a ligature model:

As we have demonstrated that the NBD-nanoparticle is capable of inhibiting the formation of the bone resorbing cells (osteoclasts) in vitro, we were interested in investigating if the nanoparticle can block the NF-κB activation-induced osteoclastogenesis leading to less severe bone loss. Thus, to examine the ability of NBD-nanoparticles to inhibit alveolar bone loss, a murine ligature-induced periodontitis model is used as previously described. Microinjections of NBD-nanoparticles, nanoparticles or PBS was done. In addition, they were infected with *P. gingivalis* every other day for 3 times. Control mice had sham-ligature and sham-infection. Seven days' post ligation, the mice were euthanized and the skulls were harvested. Measurement of CEJ- ABC distance was done morphometrically with the help of microscopy (Fig.15) and Micro-CT scan (Fig. 16) at six different sites.

Our results showed that there was no significant difference in the CEJ-ABC distance in the non-ligated group. Conversely, the ligated mice treated with empty nanoparticles or no treatment exhibited more CEJ-ABC distance. However, the NBD-nanoparticle treated mice showed a lesser CEJ-ABC measurement.

Subsequently, we determined the bone loss, which was obtained by subtracting the total CEJ-ABC distance of ligated to that of unligated mice (Fig. 17). Our results demonstrated that the bone loss was significantly reduced after treatment with the NBD-nanoparticles when compared to that of sham mice.

These results indicate that the NBD-nanoparticles markedly reduced the bone loss in a murine ligature induced periodontitis model. This observation supports our hypothesis that the NBD-nanoparticles through inhibition of NF-κB pathway prevents the osteoclastogenesis thus reducing the bone loss.

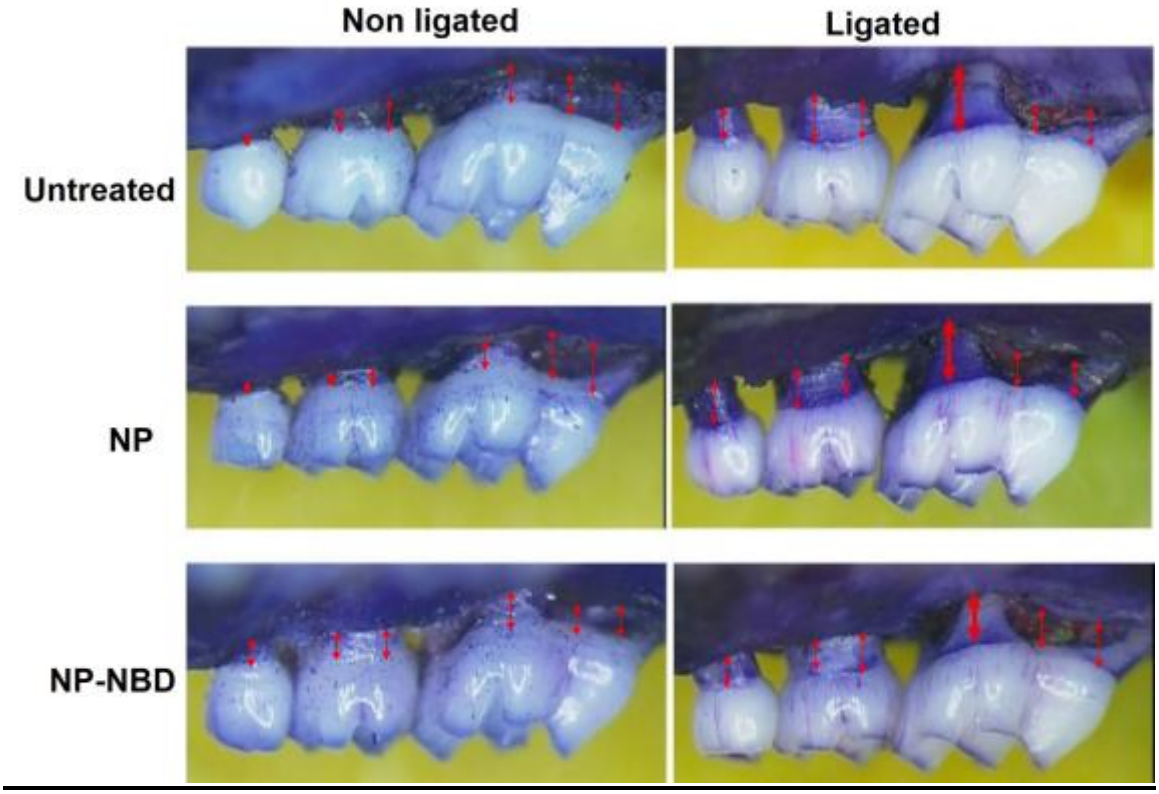

Figure 15. NBD-nanoparticle inhibits alveolar bone loss in a ligature model: C57Bl/6 mice were ligated around 2nd molar to induce bone loss. The mice were treated with NBD-NP, empty NP, or left untreated. The mm distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) was measured at 6 most affected maxillary buccal sites and the readings were totaled for each mouse. Pictures were captured using a Nikon Digital Sight DS-U3 camera controller (Nikon Instruments Inc.)

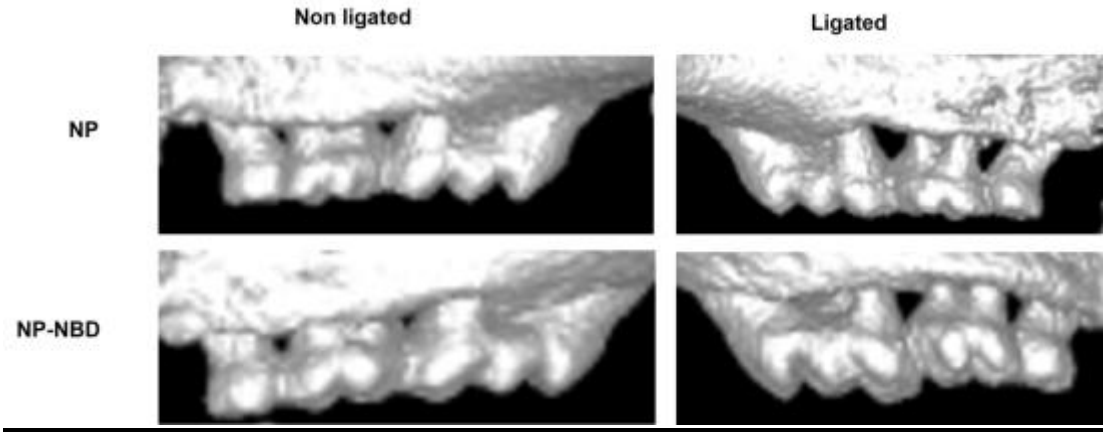

Figure 16. NBD-nanoparticle inhibits alveolar bone loss in a ligature model: C57Bl/6 mice were ligated around 2nd molar to induce bone loss. The mice were treated with NBD-NP, empty NP, or left untreated. The mm distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) was measured at 6 most affected maxillary buccal sites and the readings were totaled for each mouse.

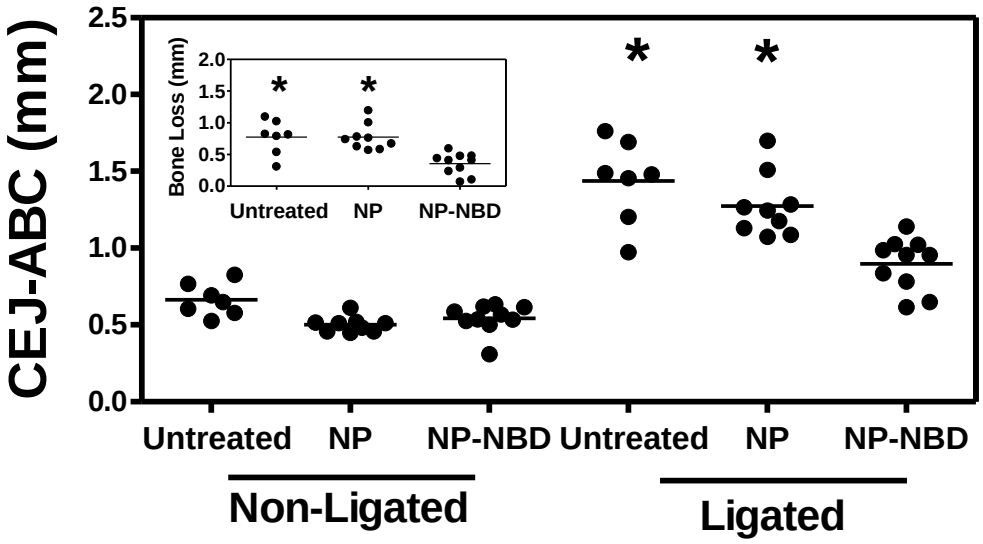

Figure 17. NBD-nanoparticle inhibits alveolar bone loss in a ligature model. C57Bl/6 mice were ligated around 2nd molar to induce bone loss. The mice were treated with NBD-NP, empty NP, or left untreated. The mm distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) was measured at 6 most affected maxillary buccal sites and the readings were totaled for each mouse. The data are means ± SD (*n* = 7 for untreated ligated and non-ligated, 9 for NP ligated and non-ligated, 10 for NBD-NP ligated and non-ligated mice). The CEJ-ABC reading of each mouse was represented by each dot. Bone loss was calculated by subtracting the six-site total CEJ-ABC distance for the ligated mice from the six-site total CEJ-ABC distance of the unligated mice. Asterisks indicate statistically significant ($p < 0.05$) differences between NBD-NP treated and other groups.

CHAPTER 4: DISCUSSION

Periodontitis is characterized by both gingival inflammation and alveolar bone loss. NF-κB signaling pathway is pivotal in osteoclastogenesis and infection-induced pro-inflammatory responses for being at the crossroad of a variety of signaling pathways. *Porphyromonas gingivalis* (*P. gingivalis*) is one of the most important and widely investigated periodontal disease etiological pathogens. Reasons for a being an extensively studied organism in periodontitis might range from being a key stone pathogen, member of red complex, persistence of the species in periodontal patients to easy culture methods and genetic manipulation. In addition, inoculation of *P. gingivalis* in animals induces gingival inflammation and alveolar bone loss. Because of their critical role in host immune responses, NF-κB must be tightly regulated to avoid excessive or defective immune responses. Many authors have described NF-κB as “holy grail”, “master switch”, “master regulator”, “center piece” of inflammatory responses (Abu-Amer, 2013; Firestein, 2004; Lawrence, 2009). As the evidence shows NF-κB to be a key player in the inflammation, the pathway has been a therapeutic target for many researchers.

A nanoparticle cargo carrier undergoing FDA-approved clinical trials has been linked with therapeutic peptide, Nemo Binding Domain (NBD) inhibitory peptide, which prevents NF-κB activation. A peptide-nanoparticle complex is formed and inhibition of NF-κB signaling has been demonstrated. We hypothesized that

the NBD-nanoparticles can lead to inhibition of osteoclastogenesis directly and pro-inflammatory cytokines production in vitro. We also hypothesized that this nanoparticle inhibits gingival inflammation and prevents bone loss in a murine model for periodontitis.

A right balance between the bone forming osteoblasts and bone resorbing osteoclasts is of utmost importance for the bone homeostasis and maintenance. When the balance tips towards formation of excessive osteoclasts, it results in bone resorption. Because periodontal disease is characterized by bone loss and the NF-κB activation leads to formation of bone resorbing cells, we wanted to establish the ability of the NBD-nanoparticles to prevent RANKL-induced osteoclastogenesis. Our results demonstrated a decrease in the number of TRAP-positive cells after treatment with the NBD-nanoparticles confirming that the NBD-nanoparticle is able to inhibit RANKL-induced osteoclastogenesis by blocking the NF-κB pathway (Fig.11). Furthermore, we wanted to investigate the ability of this nanoparticle to inhibit pro-inflammatory cytokine production. Since, periodontal disease is a repercussion of chronic inflammation and the NF-κB pathway has a central role in the production of cytokines especially those that are implicated in periodontitis, we examined the expression of these cytokines- IL-1β, IL-6 and TNFα (Fig. 12,13). Our data showed a marked reduction in the levels of cytokines and suggested that the NBD-nanoparticles are effective in inhibiting the pro-inflammatory cytokine production by blocking the NF-κB.

After establishing the action of NBD-nanoparticles in a set of *in vitro* experiments, we studied the ability of these nanoparticles *in vivo.* For this purpose, we used a murine ligature-induced periodontitis model and additionally infected them with *P. gingivalis* to exacerbate the gingival inflammation. Our results were consistent with the *in vitro* experiment data and demonstrated a decrease in the expression of inflammatory cytokines important in the pathogenesis of the periodontal disease (Fig. 14). Similarly, the NBD-nanoparticle treated group showed less CEJ-ABC distance when compared to the untreated (Fig. 17). This data suggests that the nanoparticles are effective in inhibiting gingival inflammation and alveolar bone loss in a murine ligature-induced periodontitis model.

Normally, in response to an infection, the IKK complex is activated by the binding of IKKγ to the IKKα and IKKβ subunits. This leads to the phosphorylation of the IkB rendering the free NF-κB to translocate to the nucleus thus initiating the expression of genes involved in the production of pro-inflammatory cytokines and osteoclastogenesis. The nanoparticle cargo delivers the NBD inhibitory peptide in to the cell and the peptide prevents the binding of IKKγ to the Nemo binding domain, thus blocking the downstream pathway that leads to the production of NF-κB. This results in the prevention of inflammation and bone loss.

Future studies might be directed at testing the nanoparticles using the other bacteria associated with the pathogenesis of the disease. Indeed, this study might

provide data on differential expression of cytokines by different bacteria and also the amount of suppression of the inflammation and bone loss by the nanoparticles. Also, nanoparticles with another peptide as a control can be used to check if the action is caused by the NBD nanoparticles. Characterization of the cell types that are involved in cytokine production is another direction of future studies. As the nanoparticle can be used as a drug delivery vehicle for other peptides, other processes of the NFkB pathway can be blocked.

In summary, our experimental results have indicated that this novel peptide-nanoparticle complex is effective to inhibit pro-inflammatory cytokine production, and RANKL-induced osteoclastogenesis. This nanoparticle inhibits pro-inflammatory cytokine expression in gingivae and prevents alveolar bone loss in a murine model for periodontitis. This novel nanoparticle showed significant therapeutic potential to cure periodontitis and other inflammatory diseases.

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- Exploring a novel NF-κB-inhibiting Nanoparticle for Periodontitis therapy
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