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The Characterization of Structure and Biologic Activity of Novel Lipids from Porphyromonas endodontalis

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The Characterization of Structure and Biologic Activity of Novel Lipids from
Porphyromonas endodontalis

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Introduction

The significance of the role of microorganisms in the pathogenesis of pulpal and periradicular disease has been well established by several distinguished studies (Kakehashi *et al*, 1965; Sundqvist, 1976; Patterson, 1976; Moller, 1981). Infections of the pulp initially produce an inflammatory response within the pulp that often results in complete pulpal necrosis and subsequently, in the periradicular region, results in the formation of a lesion characterized by local bone destruction (Bergenholtz, 1990). The microorganisms isolated from the infected root canal system have been extensively studied and shown to be predominantly Gram-negative and strictly anaerobic (Fabricius, 1982; Farber and Seltzer, 1988). As with the bacteria themselves, specific virulent byproducts that bacteria release into their surrounding environments have been studied for possible effects on immune-inflammatory events (Dahlen and Bergenholtz, 1980; Schonfeld et al, 1982; Yamasaki et al, 1992). Recently, the most studied of these bacterial virulence factors is the endotoxin lipopolysaccharide (LPS). LPS is a major component of the outer membrane of Gram-negative microorganisms. This complex glycolipid has been shown to be a potent stimulant of inflammation (Raetz, 1990; Cohen, 2002) and endodontic pathogenesis (Dwyer and Torabinejad, 1981). Interest in additional bacterial virulence factors, namely specific complex lipids associated with persistent periodontal disease has increased due to the significant findings related to LPS and formation of

endodontic periradicular lesions. Recently, several of these lipids have been

structurally characterized and are now known to be produced by a number of

phylogenetically related periodontal pathogens including *Porphyromonas*

gingivalis (Nichols and Rojanasomsith, 2006), *Prevotella intermedia* and

Tannerella forsythia. Results have demonstrated a possible correlation between

the presence of these complex lipids from *P.gingivalis* and the increased

production of pro-inflammatory cytokines from cells responsible for bone

remodeling (Nichols and Rojanasomsith, 2006). Researchers have identified

specific cytokines produced by both inflammatory and non-inflammatory host

cells involved in the bone resorptive process (Stashenko, 1994; Fouad, 1997;

Stashenko, 1998). These cytokines include IL-1α, IL-1β, TNF-α, IL-6, and IL-11

(Stashenko, 1994; Manolagas, 1995; Kawashima and Stashenko, 1999).   At

least one phylogenetically related organism, *Porphyromonas endodontalis*, is

recovered in diseased pulp canals (van Winkelhoff *et al*, 1985; van Winkelhoff *et*

al, 1992; Siquiera *et al*, 2006). It is postulated that this microorganism produces

similar complex lipids that may induce and maintain immune-mediated

periradicular destruction in endodontic lesions.

     Porphyromonas endodontalis is a Gram-negative bacterium frequently

recovered in necrotic root canals (van Winkelhoff *et al,* 1985; Siquiera *et al,*

2006). *P. endodontalis* is believed to contribute to the immune-activated

destruction occurring during apical periodontitis via release of noxious

byproducts including LPS but there is a possibility that other bacterial products

may be involved, namely phosphorylated dihydroceramide lipids. Examination of

the complex lipids synthesized by *P. endodontalis* for their potential role in activating host cells responsible for apical periodontitis has not occurred. We hypothesize that the phosphorylated dihydroceramide lipids of *P. endodontalis* play a significant role in stimulating immune-mediated inflammatory cells as well as promoting maturation and activation of osteoclasts resulting in periapical inflammation and bone destruction. Thus, this study aimed to investigate which complex lipid are produced by the endodontic pathogen, *P. endodontalis,* and the potential role these lipids play in the promotion of osteoclast activation and formation of the periapical endodontic lesion.

Review of Literature

Etiology of Apical Periodontitis

Apical periodontitis is characterized as local inflammatory destruction of the bone and the periodontium around the apex of a tooth in response to an infection of endodontic origin (Moller *et al.* 2004). While some experiments have demonstrated chemical and physical factors as inducers of a periradicular inflammatory response, evidence for the essential role of microbial agents in dental pulp infection and the pathogenesis of apical periodontitis is extensive and well-established (Kakehashi, 1965; Sundqvist, 1976; Moller, 1981).

The presence of bacteria in the necrotic dental pulp of teeth with associated pathologic conditions was reported more than a century ago, when Miller (1894) hypothesized that microorganisms are the causative agents of endodontic pathosis (Miller, 1894). Yet, it was not until over a half century later, when the casual relationship between periapical inflammation and bacterial infection was convincingly demonstrated. In the classic study by Kakehashi *et al.* (1965), it was shown that pulpal necrosis and periapical inflammation developed in rats subjected to mechanical pulp exposure and kept exposed to the conventional microbial environment of the oral cavity. In contrast, germ-free animals demonstrated minimal pulpal inflammation and no periapical destruction, and formed reparative dentin at the exposure sites, exhibiting the substantial healing capability of the pulp in the absence of microbial infection (Kakehashi,

1965). Subsequent animal and human studies confirmed the cause and effect relationship between pulpal infection and periapical pathology. Sundqvist, in his 1976 thesis, studied thirty-two traumatized incisors with pulp necrosis, yet clinically intact crowns. Eighteen of the nineteen (95%) teeth with associated periapical lesions presented with positive cultures for bacteria that were predominantly strict anaerobes, while no bacterial were cultivated from the thirteen teeth without periapical lesions (Sundqvist 1976). In a well-controlled monkey experiment in which the pulps of twenty-six teeth were rendered necrotic under sterile conditions and immediately sealed in the uninfected root canals, none developed apical periodontitis, even after 6-7 months of observation. In distinction, forty-seven of the fifty-two (90%) teeth in which the pulps were deliberately infected prior to sealing of the access opening, exhibited periapical destruction (Moller et al. 1981). This study demonstrated that apical periodontitis cannot be caused by necrotic pulp tissue alone, via toxic tissue breakdown products, but that the presence of bacteria within the pulp appears to be an absolute requirement for the development of periradicular pathosis.

Studies on Endodontic Flora and Modes of Entry

Microorganisms may gain entry into the dental pulp complex in one of several routes. The most common pathway may be through openings in the dental hard tissues as a result of the caries process, operative procedures, and trauma-induced micro-fractures within the tooth structure, all of which provide an established route of entry for microorganisms to invade. Anachoresis, which is

the localization and fixation of blood-borne bacteria to zones of inflammation, has also been proposed in cases where teeth with necrotic pulps and clinically intact crowns were observed (Robinson and Boling, 1941; Gier and Mitchell, 1968; Tziafas 1989). However, this theory remains controversial and the extent to which this process may occur is unknown (Delivanis and Fan, 1984; Siquiera, 2002). An additional pathway for bacterial contamination and infection of the pulp may be through exposed dentinal tubules or accessory canals found at the cervical root surface in the periodontal pocket, although the importance of this pathway has also been disputed (Seltzer *et al.* 1963; Mazur and Massler, 1964).

There are over 500 bacterial species currently recognized as normal inhabitants of the oral cavity and an additional 200 may still be detected through molecular methods of identification (Moore and Moore, 1994; Munson *et al.,* 2002; Dewhirst and Paster, 2005). Although all bacterial species have the theoretic potential and capability of invading and infecting the pulp space, inducing periradicular inflammation and bone destruction, only a small percentage of species have been consistently isolated from such root canal systems (Sundqvist, 1994).

The necrotic root canal system provides a unique and most often favorable ecological niche for specific bacterial species. Decreased oxygen tension, availability of host tissues, and inflammatory exudates produced at the bacteria-inflammatory tissue interface are the primary nutritional factors selecting for bacterial growth (Sundqvist, 1992). Previous experiments have shown that

the relative proportion of obligate anaerobes increases and that of facultative bacteria decreases, with time elapsed after infection (Fabricius *et al.,* 1982; Tani-Ishi *et al*, 1994). Bacteriologic sampling of necrotic root canals of chronically infected teeth with primary apical periodontitis indicates that the microbial population is predominantly anaerobic and Gram-negative (Bergenholtz, 1974; Sundqvist, 1976). Most of these species have also been identified in periodontal pockets (Moore, 1987; Socransky *et al.,* 1988). The anaerobic infection is most often polymicrobial, harboring anywhere from two up to eight species, with no more than twenty species having ever been found in one root canal (Dahlen and Haapasalo, 1998). Common bacterial genera that have been isolated in both human and animal studies include *Fusobacterium, Streptococcus, Prevotella, Eubacterium, Peptostreptococcus, Camphylobacter, Porphyromonas,* and P*ropionibacterium* (Sundqvist, 1994).

Bacterial interactions play a significant role in determining the composition and pathogenicity of the root canal flora. Virtually all microbes that are present in root canals have the potential to initiate a periapical inflammatory response and bone destruction (Sundqvist, 1976). However, some combinations have a tendency to associate together, providing for a mutually supportive (synergistic) environment, whereas others may create negative (antagonistic) associations by competing for nutrients, thus inhibiting the growth of other bacteria (Greiner and Mayrand, 1986; Sundqvist 1992). In studies conducted by Fabricius *et al.*, bacteria originally isolated from the root canals of monkeys with periapically involved teeth were then inoculated in various combinations or as individual

strains into the root canals of other monkeys. Infection of root canals with individual bacterial species produced a relatively mild periapical inflammation and little periapical destruction, whereas in combinations, the same bacterial species were highly pathogenic and capable of inducing more severe periapical reactions and larger areas of periapical destruction (Fabricius *et al.,* 1982b). These observations and later studies have confirmed that the virulence of individual species may vary considerably and highlight the importance of microbial synergy for the pathogenicity of the endodontic flora (Dahlen *et al.,* 1987). The pathogenic properties of these microorganisms are affected by a variety of factors which include: (1) the presence in sufficient numbers to initiate and maintain infection, (2) the ability to acquire nutrients from the host tissue for growth and survival, (3) the ability to evade, withstand, and/or alter host defenses, and (4) the synthesis and release of a variety of virulence factors including proteolytic enzymes, metabolites, and cell wall components—such as endotoxin (lipopolysaccharide, LPS) from Gram-negative bacteria and proteoglycans from Gram-positive bacteria--into the periapical area where they are capable of damaging host tissues (Horiba *et al.,* 1991; Hashioka *et al.,* 1994). LPS, in particular, is the best studied of these virulence factors, and has also been identified as a highly potent initiator of inflammation and a major factor in bone resorption (Dwyer and Torabinejad, 1981; Dahlen *et al.,* 1981; Schonfeld *et al.,* 1982).
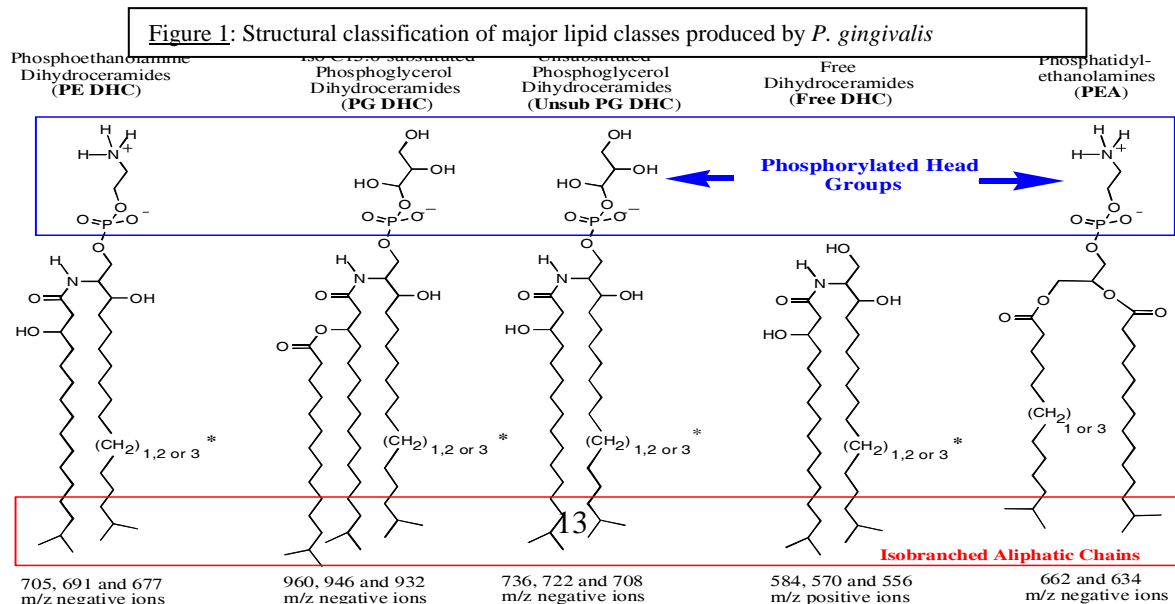
***Porphyromonas endodontalis:* Characteristics & Identification in Necrotic Root Canal Systems**

At the present, no definitive documentation exists which identifies a single microbial species responsible for the pathogenesis of periradicular diseases. Evidence suggests that acute periradicular abscesses may be associated with particular microbial species (Sundqvist et al. 1979; van Winkelhoff et al. 1985; Brook *et al.* 1991). In a study by Brook et al., cultured aspirates of pus from periradicular abscesses resulted in a predominance of black-pigmented anaerobic rods. *Porphyromonas endodontalis* is a Gram-negative, obligately anaerobic, non-spore forming bacteria which appears as a non-motile pleomorphic rod or coccobaccili. *P. endodontalis and P. gingivalis* have been closely associated with acute symptoms of endodontic infections (Haapasalo *et al.* 1986; Sundqvist *et al.* 1989). A study conducted by van Winkelhoff et al. found that all abscesses of endodontic origin examined harbored one or more of the genera *Prevotella* and *Porphyromonas*, with *Porphyromonas endodontalis* found in 53% of the samples. The mechanisms of pathogenicity of *Porphyromonas endodontalis* are not well understood. It has been postulated that the release of outer membrane blebs consisting of complex lipids including lipopolysaccharide, may be an important virulence factor involved in the pathogenesis of the periradicular lesion (Haapasalo 1989). Bacterial lipopolysaccharide (LPS) is an endotoxin and a major component of the cell wall

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of Gram-negative microorganisms. Several studies have shown it to be a potent

microbial initiator of inflammation (Raetz, 1990; Cohen, 2002) and endodontic

pathogenesis (Dwyer and Torabinejad, 1981). A positive correlation has been

reported between the levels of LPS in root canals and the presence of periapical

lesions (Dahlen and Bergenholtz, 1980; Schonfeld *et al.* 1982). To date, few

studies have examined the remaining lipid classes that exist on the outer

membrane of Gram-negative bacteria associated with endodontic infections.

Identification of Novel Lipid Classes Produced by Gram-negative Bacteria

Several species of Gram-negative bacteria have been shown to

synthesize an array of phosphoethanolamine and phosphoglycerol

dihydroceramides that share a core lipid structure consisting of three long chain

bases in amide linkage to 3-OH (*iso)* $C_{17:0}$ (Nichols 1998; Nichols *et al.* 2004). *P.*

gingivalis produces novel classes of lipids that are more than structural

membrane lipids. These lipid classes are depicted below (Figure 1) and the

characteristic ions for each lipid class are listed at the bottom of each chemical

structure.

Figure 1: Structural classification of major lipid classes produced by *P. gingivalis*

Phosphoethanolamine Dihydroceramides (PE DHC)	Iso C17:0 substituted Phosphoglycerol Dihydroceramides (PG DHC)	Unsubstituted Phosphoglycerol Dihydroceramides (Unsub PG DHC)	Free Dihydroceramides (Free DHC)	Phosphatidyl-ethanolamines (PEA)
705, 691 and 677 m/z negative ions	960, 946 and 932 m/z negative ions	736, 722 and 708 m/z negative ions	584, 570 and 556 m/z positive ions	662 and 634 m/z negative ions

*   The long chain bases vary from 17 to 19 carbons in length. The 17 and 19 carbon long chain bases are isobranched as shown but the 18 carbon long chain base is a straight aliphatic chain.

Each of these lipid classes contains iso-branched aliphatic chains, either as fatty acids or as long chain bases. Both phosphorylated dihydroceramide classes of *Porphyromonas gingivalis* were shown to promote prostaglandin secretory responses in gingival fibroblasts in culture and the phosphoglycerol dihydroceramides caused substantial morphologic changes in gingival fibroblasts, suggesting either apoptosis, cell necrosis or both (Nichols *et al.* 2004). The ability of these lipids to produce a biological response supports their classification as microbial virulence factors.

Lipid extracts from *Prevotella intermedia*, *Tannerella forsythia* and *Bacteroides fragilis* are of interest. Substituted phosphoglycerol dihydroceramide lipids are recovered as 960, 946 and 932 m/z ions when analyzed by electrospray-MS. Comparison of the mass spectra for lipids recovered from these species shows that *Porphyromonas gingivalis* is most prolific in producing the phosphoglycerol dihydroceramide lipids followed by *Tannerella forsythia* and finally *Prevotella intermedia* and *Bacteroides fragilis* are minimally capable of synthesizing this lipid class (Nichols *et al.,* 2004). *Porphyromonas endodontalis* is expected to produce these phosphoglycerol dihydroceramides as well as an array of phosphoethanolamine dihydroceramide lipids in a manner similar to *Porphyromonas gingivalis*.
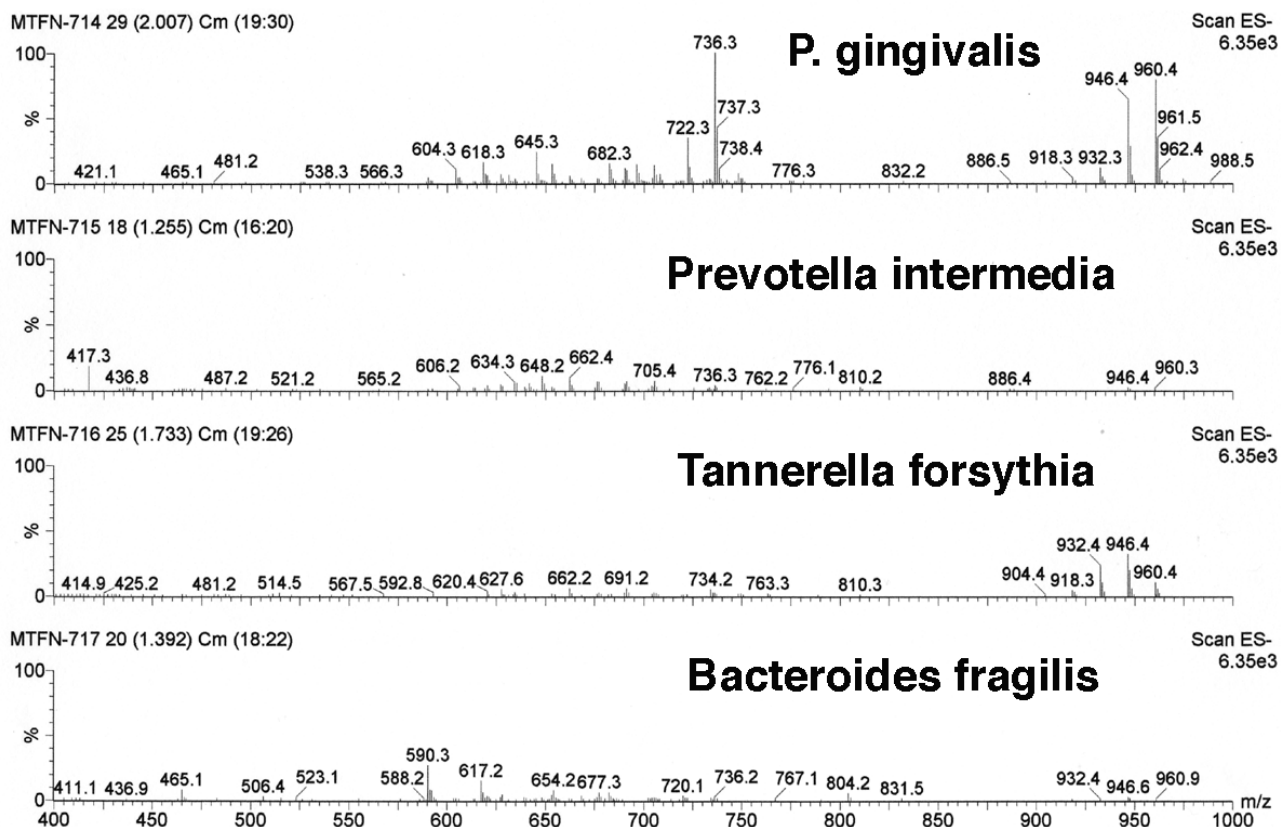
Figure 2: Total lipid profiles from various Gram-negative bacteria

Biological effects of Dihydroceramide Lipids

Dihydroceramide lipids of *P. gingivalis* and other phylogenetically related organisms have been identified in lipid extracts of diseased tooth roots and diseased gingival tissues (Nichols, 1998). Recent studies have shown significant pro-inflammatory events that have been induced by the presence of these novel lipids. More specifically, when gingival fibroblasts were treated with substituted phosphoglycerol dihydroceramide lipids isolated from *P. gingivalis*, significant

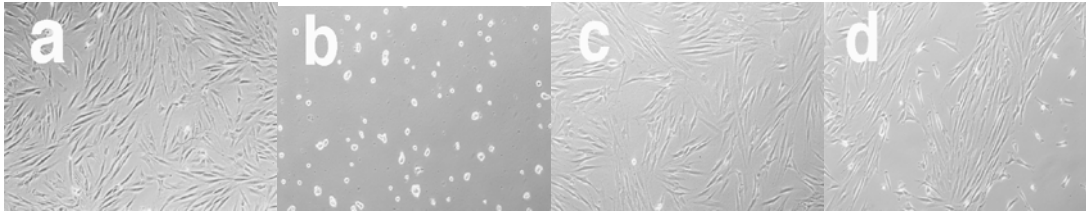morphological changes were seen as shown below (figure 3) in frame b.

Figure 3: Morphological changes seen in gingival fibroblasts when exposed to various lipid fractions from *P. gingivalis*

Cell shrinkage and detachment from the culture dish is evident after only 6 to 8 hours of exposure to this lipid class. The phosphoethanolamine dihydroceramide lipids of *P. gingivalis* had no effect on cell morphology (frame c) and the total lipid extract of *P. gingivalis* only minimally affected fibroblast morphology (frame d). Chemical removal of the substituted fatty acid from the phosphoglycerol dihydroceramide lipid class (producing the unsubstituted phosphoglycerol dihydroceramide lipids) completely eliminated the deleterious effect of this lipid class on gingival fibroblast morphology. These results show the direct effect of phosphoglycerol dihydroceramide lipids on gingival fibroblast morphology when cells are exposed to these lipids in culture.

Gingival fibroblasts were treated with *P. gingivalis* lipids as previously described and after 2 hours of exposure to the respective lipid classes, the cultures were treated with human recombinant interleukin-1β (20ng/ml). After 24 hours, the culture media samples were harvested and analyzed for prostaglandin levels by GC-MS. The following figure (figure 4) shows that the substituted phosphoglycerol dihydroceramide lipids markedly potentiated the effects of IL-1β on prostaglandin secretion from gingival fibroblasts. The phosphoglycerol

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dihydroceramide lipids had the most pronounced effect on prostaglandin E_2. Prostaglandin E_2 is known to promote vasodilation, inflammatory responses and to stimulate osteoclast mediated bone resorption. The total lipid extract of *P. gingivalis* promoted IL-1β responses only to about half the extent of the phosphoglycerol dihydroceramide lipids and the phosphatidylethanolamine dihydroceramide lipids only slightly promoted prostaglandin release. The phosphoethanolamine dihydroceramide lipids promoted IL-1β -mediated prostaglandin release only to a slight extent and the unsubstituted phosphoglycerol dihydroceramide lipids did not promote IL-1β -mediated prostaglandin secretion. These results demonstrate that the substituted phosphoglycerol dihydroceramide lipids of *P. gingivalis* promote inflammatory reactions by potentiating the effects of IL-1β -mediated responses in gingival fibroblasts. Chronic inflammatory responses associated with periodontal or endodontic disease sites are associated with elevated levels of IL-1β and prostaglandins within the diseased tissues.
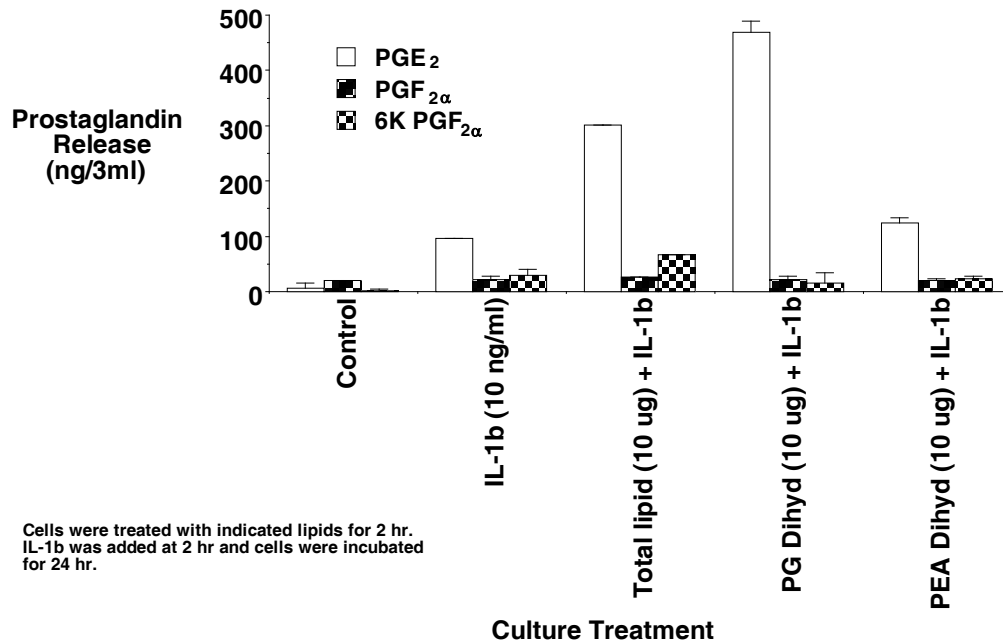
Figure 4: Graph displaying PGE2 release from gingival fibroblasts after being exposed to various lipid fractions from *P. gingivalis*

In the study conducted by Nichols *et al*., RAW 264.7 cells were treated with the substituted phosphoglycerol dihydroceramide (PG DHC) lipids and/or RANKL in a manner similar to the fibroblasts described above. Cells were incubated for three days after which cells were stained for actin (green) and nuclei (yellow-Figure 5). Control cells or cells treated with *P. gingivalis* LPS failed to undergo cell fusion. However, cells treated with substituted PG DHC lipids showed marked cell fusion in a manner very similar to that produced with exposure to RANKL. Combining PG DHC lipids with RANKL promoted an even greater fusion of RAW cells to form multinucleated giant cells. Additional experiments showed that the soluble antagonist of RANKL, called osteoprotegrin (OPG), did not affect cell fusion for cells treated with PG DHC lipids but completely inhibited RANKL-mediated cell fusion (data not shown). This result

indicates that PG DHC lipids mediate their effects on osteoclast formation

independent of RANKL.   Additional experiments reveal that osteoclasts exposed

to PG DHC lipids will promote bone resorption or formation of resorption pits on

dentin slices. Altogether, these results demonstrate that a major lipid class of *P.*

gingivalis will damage fibroblasts in culture, will promote prostaglandin secretory

responses and will also promote bone destruction independent of RANKL

(Nichols *et al.*, 2008). The primary goal of this experimental procedure is to

determine whether this same lipid class is produced by *P. endodontalis* and

whether the recovered lipid is capable of producing the same biological

responses as those shown in figure 5 below.

Med   Pg-LPS

PGDHC   RANKL   Both
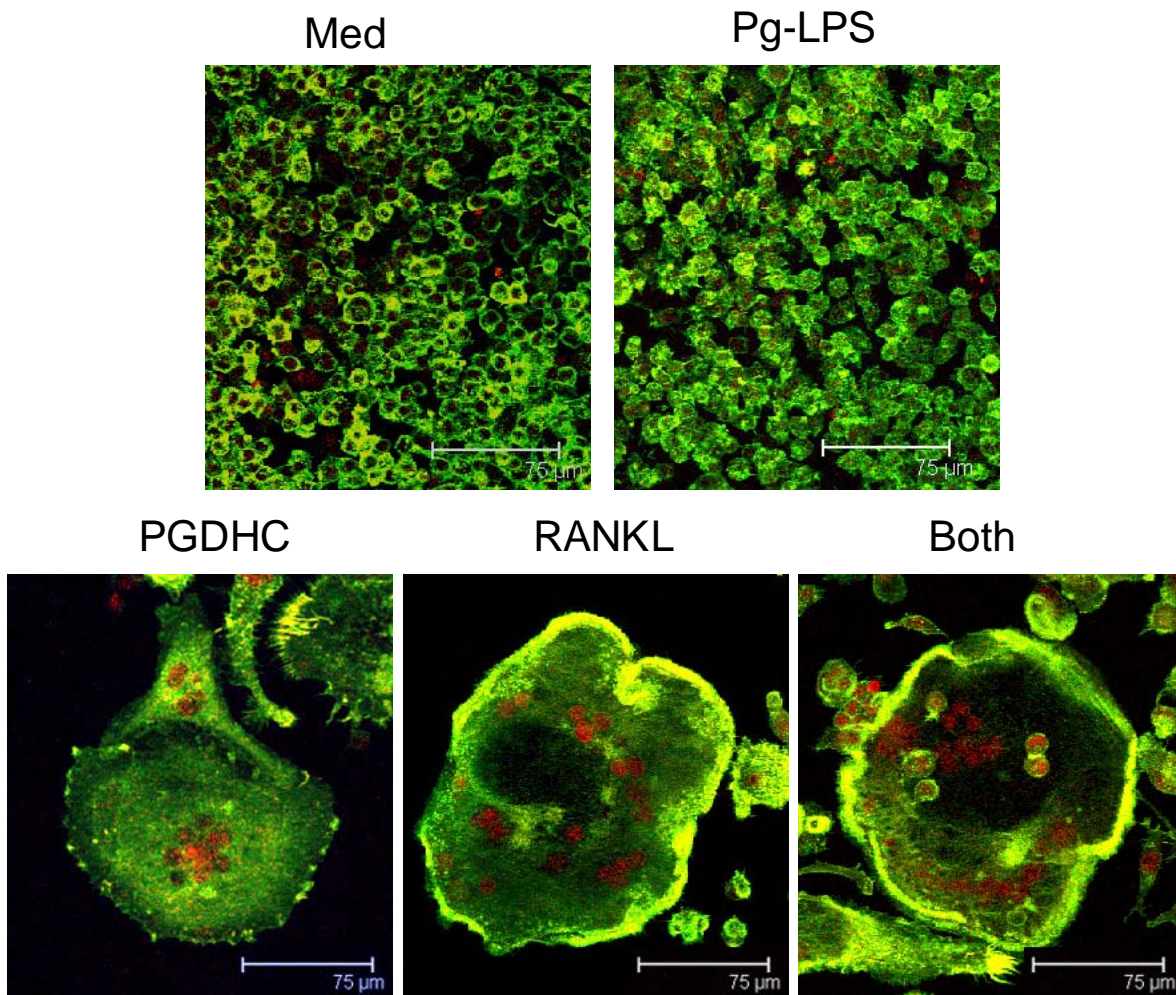
<u>Figure 5</u>: Induction of osteoclast differentiation when exposed to PG DHC isolated from *P. gingivalis*

Osteoblast Cell Origin, Plasticity and Function

Osteoblasts are derived from primitive mesenchymal cells (Aubin & Lui,

2002). During embryonic skeletogenesis, they participate in two distinct modes

of bone development, intramembranous and endochondral bone formation

(Marks & Odgren, 2002). Each pathway involves the osteoblasts' specialized

function of secreting osteoid to either form bone spicules, as in intramembranous

formation or by secreting osteoid onto a pre-formed cartilage matrix, as observed

in endochondral bone formation.   Mesenchymal cells located on periosteal

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surfaces and within bone marrow stroma and provide a source of osteoblasts which act in concert with osteoclasts to remodel bone during growth and maintain bone structure during adulthood (Mackie, 2003).

Even though osteoblasts are classified as specialized cells, they are not terminally differentiated. Some osteoblasts ultimately become embedded in bone matrix to form osteocytes, which gradually stop secreting osteoid. These cells reside in lacunae and form a network throughout mineralized bone tissue, communicating through gap junctions with each other and surface osteoblasts (Marks & Odgren, 2002).   Other osteoblasts, however, have a different fate, undergoing apoptosis rather than becoming embedded in bone matrix (Jilka, 1999).

Proliferation and differentiation of cells of the osteoblast lineage occur under the influence of a number of transcription factors, growth factors and hormones (Aubin & Lui, 2002). Transcription factors such as Cbfa-1/RunX2 are necessary for the expression of osteoblast-specific genes, such as osteocalcin (Mackie, 2003). Growth factors such as transforming growth factor-beta (TGF-β) and bone morphogenic protein (BMP) are produced by osteoblasts and sequestered in bone matrix; it is thought that they are released and made available to osteoblast precursors in active form as a result of the osteoclastic resorption of bone matrix (Mackie, 2003).

Osteoblasts play a significant role in creating and maintaining skeletal architecture, and they do this in two ways. They are responsible for deposition of bone matrix and they regulate the differentiation and activity of the bone-

resorbing osteoclasts. Also, as a result of their ability to regulate osteoclastic

activity, osteoblasts indirectly play an important role in calcium homeostasis

(Mackie, 2003). As osteoblasts differentiate from their precursors they begin to

secrete bone matrix proteins. Type I collagen is the major protein in bone matrix,

representing about 90% of the organic matrix. The network of type I collagen

fibers provides the structure on which bone mineral is deposited (Mackie, 2003).

Osteoblasts also secrete non-collagenous proteins, including proteoglycans,

glycoproteins and carboxylated proteins (Robey, 2002). Osteoblasts also have

an indirect role in the mineralization of osteoid. Bone mineral consists of crystals

of hydroxyapatite. Initiation of crystal formation is almost certainly assisted by

macromolecular 'nucleators', namely candidates for which are the osteoblast-

derived phosphate-containing proteins of bone matrix such as bone sialoprotein

(Mackie, 2003). Osteoblasts also contribute to the process of mineralization

through provision of enzymes that regulate phosphoprotein phosphorylation,

including alkaline phosphatase, a membrane-bound marker of osteoblast

differentiation (Mackie, 2003).

Regulation of Osteoclast Function

Osteoclasts are large, multinucleated specialized giant cells responsible

for bone resorption that arise from a hematopoietic stem cell lineage of

monocytes and macrophages (Suda *et al.,* 1992). Osteoclastic bone resorption

consists of numerous steps and studies have identified several key elements

thought to be important for osteoclast differentiation (osteoclastogenesis) and

activation and function (Teitelbaum and Ross, 2003). These include nuclear

factor of activated T-cells (NFATc1), receptor activator of nuclear factor kappa B

ligand (RANKL), cathepsin K (CSK), tartrate resistant acid phosphatase (TRAP),

tumor necrosis factor-β (TNF-β), interferons (IFNs), and interleukins (ILs). Of

these factors, NFATc1 and RANKL are thought to play an essential role in

osteoclast development (Horsley et al., 2002; Boyle *et al.,* 2003). In bone,

RANKL is primarily expressed on the surface of osteoblasts and bone marrow

stromal cells and lymphocytes, and signals through its respective receptor,

RANK (receptor activator of nuclear factor kappa B) that is found on osteoclast

precursors to stimulate osteoclastogenesis (Lacey *et al., 1998;* Yasuda *et al.,*

1998; Teitelbaum, 2000). Additionally, osteoblasts and bone marrow stromal

cells also produce a soluble inhibitory factor called osteoprotegerin (OPG), which

acts as a decoy receptor and competes with RANK, preventing it from binding to

RANKL (Suda *et al.,* 1999). Both RANKL and OPG are members of the TNF

receptor-like super family of molecules and the axis of RANKL/RANK/OPG is

considered to be essential for the development and activation of osteoclasts and

key regulator of bone formation and remodeling (Figure 6) (Wittrant *et al.,* 2004).
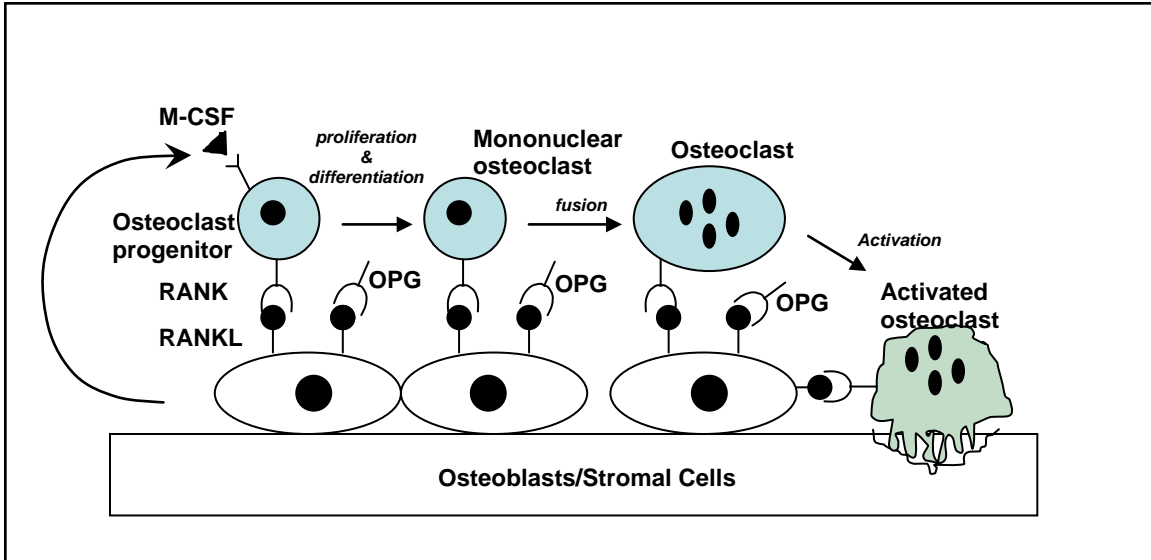
Figure 6: Schematic representation of osteoclast differentiation and activation supported by osteoblasts/stromal cells. Osteoclast progenitors of the monocyte-macrophage lineage recognize RANKL expressed by osteoblasts/stromal cells and undergo a series of differentiation stages to become activated osteoclasts responsible for bone resorption. M-CSF is another critical factor expressed by osteoblasts/stromal cells necessary for osteoclastogenesis. OPG is a soluble naturally occurring inhibitor of osteoclast differentiation that binds to RANKL, preventing its binding to RANK. Modified from Katagiri and Takahashi, 2002.

Interactions between Osteoblasts and Osteoclasts: Role in Apical Periodontitis

It has been well established that osteoblasts control the ontogeny and function of osteoclasts by producing several factors that directly bind to osteoclastic precursors (Rodan and Martin 1981). Receptor activator of NFkβ ligand (RANKL), a member of the TNF superfamily, is a membrane-residing protein on osteoblasts and their precursors, which activates receptor RANK on osteoclast precursors (Yavropoulou & Yuvos, 2008).
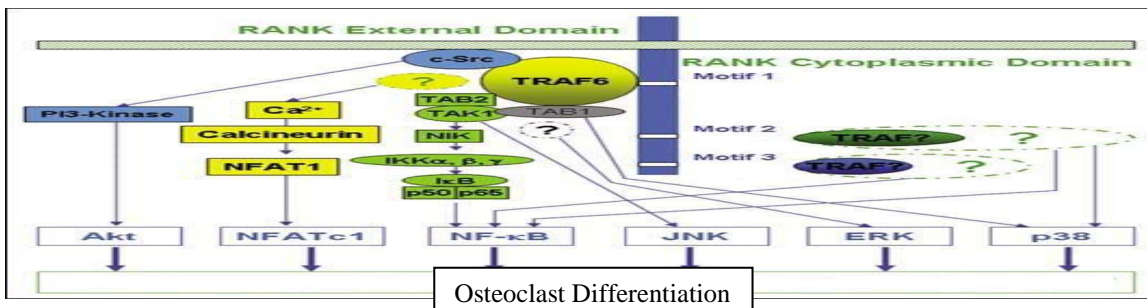
 In physiological conditions, RANKL is primarily expressed by stromal cells in bone marrow and osteoblasts in the periosteum. However, in states of inflammation involving the bone, RANKL expression can be significantly upregulated by osteoblasts in response to numerous pro-inflammatory cytokines (Yavropoulou & Yuvos, 2008). With this possibility, RANKL can be cleaved from the membrane surface to become a soluble ligand. Deletions in RANKL or RANK genes results in the absence of osteoclasts due to arrested differentiation of osteoclast progenitor cells (Mackie, 2003). The decoy receptor, osteoprotegrin (OPG), is also produced by osteoblasts. OPG acts through binding to RANKL and preventing its interaction with RANK.

Pro-inflammatory cytokine TNF-alpha has been shown to induce stromal cell expression of IL-1, which in turn up-regulates its own receptor and promotes RANKL production (Kobayashi et al., 2000). Johnston et al discovered that cells over-expressing TNF-alpha, when injected into nude mice, increased osteoclast number, as well as bone resorption (Johnston et al, 1989). The binding of TNF-alpha to its receptor, TNFR1, produces a signal transduction cascade that mimics that of RANKL-RANK interaction. For example, TNF promotes the recruitment of TRAF molecules to TNFR1, which leads to the activation of the NF-κB pathway (Nanes, 2003). RANKL activates the receptor RANK on osteoclast progenitor cells in a trimeric symmetric complex, interacting with an adaptor molecule, TNF receptor-associated factor 6 (TRAF6). Although several

TRAFs have been reported in the literature, TRAF6 seems to have a critical role in osteoclastogenesis. Despite the activation of overlapping TRAF6-dependent signaling cascades by other receptors, such as the Toll-like receptor family members (TLR), only RANKL can induce significant osteoclastogenesis (Yavropoulou & Yuvos, 2008).

Recent findings support the notion that bacterial lipopolysaccharide (LPS) and some inflammatory cytokines such as TNF-β and IL-1 may also be directly involved in osteoclast differentiation and activation through a mechanism partially independent from that of the RANKL-RANK interaction (Jimi *et al,* 1999; Suda *et al.,* 2002; Kobayashi *et al.,* 2000; Jiang *et al.,* 2006). The intracellular signaling cascade of TLR4 is similar to that of IL-1 receptors and both of these receptors have been shown to use TRAF6 as a common signaling adapter molecule (Kobayashi *et al.,* 2004). When osteoclast precursors were stimulated with both LPS and RANKL, LPS-induced osteoclast formation was observed even in the presence of OPG and IL-1 receptor antagonists (Suda *et al.,* 2002; Jiang *et al.,* 2006). Osteoclast formation was also stimulated by LPS in preosteoclast cells from mice lacking tumor necrosis factor (TNF)-receptor-I or TNF-receptor-II (Suda *et al.,* 2002). These results suggest that LPS stimulates osteoclast formation independent of RANKL, IL-1, or TNF-alpha action, and have opened new areas for exploring the molecular mechanisms of osteoclast differentiation. It is hypothesized that other bacterial lipid byproducts, including dihydroceramide lipids released from Gram-negative bacteria will show a similar ability to stimulate formation and maturation of osteoclasts.

NFATc1

The NFAT family of transcription factors includes five members. The necessary and permissive role of NFATc1 in osteoclastogenesis was first suggested by in vitro observations that NFATc1-deficient embryonic stem cells do not differentiate into osteoclasts, and that ectopic expression of NFATc1 causes bone marrow-derived precursor cells to undergo osteoclast differentiation in the absence of RANKL (Takayanagi *et al.,* 2002). NFATc1 has been shown to be the most strongly induced transcription factor gene mediated by RANKL stimulation. It has also been shown to be a key regulator of osteoclastogenesis and plays a critical role in the terminal differentiation of osteoclasts (Takayanagi *et al.,* 2002). RANKL-induced recruitment of TRAF6 results in the induction of intracellular calcium, which leads to the activation of calcineurin (Takayanagi *et al.,* 2002). Activated calcineurin then dephosphorylates and activates NFAT1, allowing it to translocate to the nucleus to form a ternary complex with c-Fos and c-Jun to stimulate NFATc1 gene expression (Figure 7) (Ikeda *et al.,* 2004). At the final stage of osteoclast differentiation, NFATc1 works in conjunction with Fos and Jun proteins to stimulate osteoclast-specific genes such as tartrate-resistant acid phosphatase (TRAP) and cathepsin K (Ikeda *et al.,* 2004). Thus it appears that the NFATc1 pathway is a crucial component of osteoclast differentiation, and investigation of this gene's regulatory activity in the presence of dihydroceramide lipids from *Porphyromonas endodontalis* may provide additional information on how these noxious lipids affect osteoclast differentiation and maturation.

Cathepsin K

Of the enzymes responsible for the resorption process carried out by osteoclasts, perhaps the most predominant is Cathepsin K (CSK). Cathepsin K is encoded by the human gene CTSK located on chromosome 1. This gene encodes a protein which is a lysosomal cysteine protease associated with the peptidase C1 protein family and is primarily expressed by osteoclasts. Being a protease, Cathepsin K shows high specificity for kinins that are involved in bone resorption. The enzyme's ability to catabolize elastin, collagen and gelatin allow it to easily break down bone and cartilage. Expression of Cathepsin K by osteoclasts is induced by specific pro-inflammatory cytokines such as IL-1, IL-6 and TNF- alpha. These cytokines are produced and excreted by inflammatory cells involved with recognizing tissue injury and stimulating repair. Once these cytokines bind to and stimulate osteoclast precursor cells, an upregulation of gene expression is noted and Cathepsin K is ultimately encoded and activated.

Tartrate-resistant Acid Phosphatase (TRAP)

Tartrate-resistant acid phosphatase (TRAP) is an iron-containing enzyme that can be found in cells regulating bone turnover as well as cells comprising the immune system. It is expressed by cells of the monohystiocytic lineage including macrophages (Minkin, 1982) and dendritic cells (Hayman *et al.,* 2001). The enzyme was originally identified by its unique cathodal mobility at pH 4 and is further defined by its resistance to inhibition by L (+) tartrate (Li *et al.,* 1970).

Osteoblasts contain two fractions of the TRAP enzyme. One that is released by monocytes and subsequently endocytosed and a second endogenous fraction that is present in an inactive form which is activated upon cleavage by cathepsins (Perez-Amodio *et al.,* 2005).

Mature osteoclasts show an intense staining for TRAP in normal bone architecture. For many years, TRAP has been used as a histochemical marker of osteoclast differentiation and maturation (Burstone, 1959). TRAP is secreted by the osteoclast during bone resorption and secretion correlates with resorptive activity (Kirstein *et al.,* 2006). In the resorbing osteoclast TRAP occurs in transcytotic vesicles involved in the transportation of matrix degradation products through the cell. It has been proposed that reactive oxygen species produced by TRAP help to destroy collagen and other proteins in these vesicles (Halleen *et al.,* 1999).

Toll-Like Receptor 2 (TLR2)

Toll-like receptor 2 (TLR2) is a single membrane-spanning non-catalytic pattern recognition receptor that recognizes structurally conserved molecules derived from microbes. These molecules are broadly shared by pathogens but are distinguishable from host molecules, and are collectively referred to as pathogen-associated molecular patterns (PAMPs). A number of ligands are known to activate TLR2, including LPS, lipoprotein and fimbriae but the first and latter only apply to *P. gingivalis*.

TLR2 is believed to function as a dimer. Though most toll-like receptors act as homodimers, TLR2 forms heterodimers with TLR1 or TLR6, each dimer having different ligand specificity. TLR2 may also depend on other co-receptors for full ligand sensitivity, such as with TLR4's recognition with LPS, such as MD-2. CD14 and LPS binding protein are known to facilitate the presentation of LPS to MD-2.
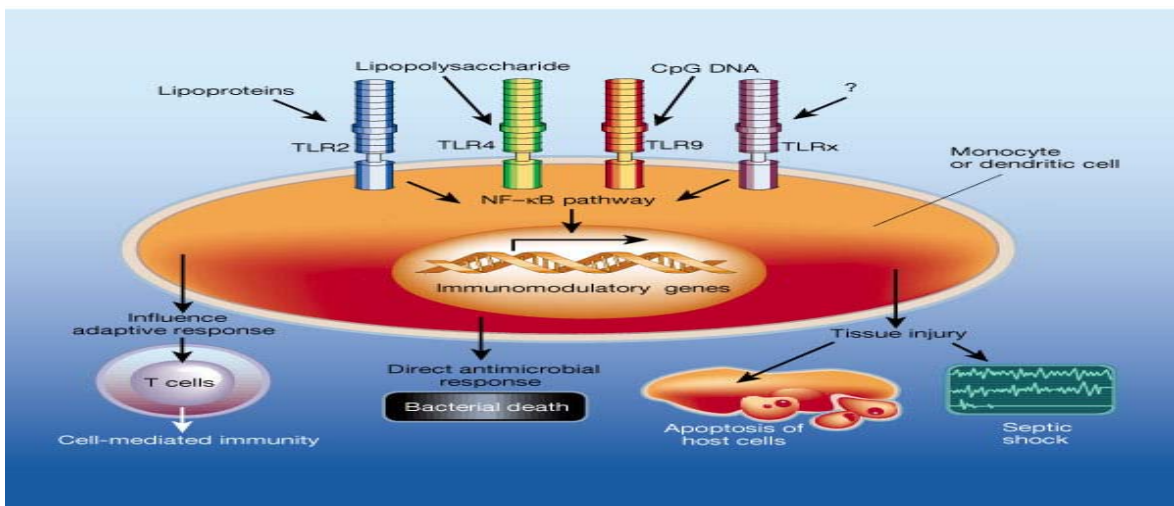

Figure 8: Schematic of various toll-like receptors including TLR2 and their effects on inflammatory response.

It has been well established that TLR2 is involved with the recognition of components from an array of microorganisms. These include Gram-positive, mycobacterial, and fungal compounds such as peptidoglycan, lipoteichoic acid, and various lipopeptides (Lien *et al.,* 1999). However, TLR2 was also originally believed to be the long sought after LPS receptor. This was based on several transfection studies that showed that expression of the mammalian TLR2 in human embryonic kidney cells that were LPS-unresponsive were now capable of responding to LPS (Kirschning *et al.,* 1998; Yang *et al.,* 1998). However, later studies revealed that

overexpression of TLR2 causes cell lines to become exceedingly sensitive to trace amounts of non-LPS lipopeptide contaminants that may have been present in the LPS preparations (Heine *et al.,* 1999; Takeuchi *et al.,* 1999; Faure *et al.,* 2000). Repurification of LPS preparations of *Salmonella* and *E. coli* clearly demonstrated that cells are activated through TLR4 as the principal signal-transducing molecule, and not TLR2 (Hirschfeld *et al.,* 2000; Tapping *et al.,* 2000). Furthermore, similar types of LPS elicited the same response in TLR2 knockout mice as in wild-type mice (Takeuchi *et al.,* 1999).

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) is a variant of polymerase chain reaction (PCR), commonly used to generate many copies of a DNA sequence, a process termed amplification. In RT-PCR, however, a RNA strand is first reverse transcribed into its DNA complement (*cDNA*) using the enzyme reverse transcriptase, and the resulting cDNA is amplified using traditional PCR.

The two-stage RT-PCR process for converting RNA to DNA and subsequent PCR amplification of the reversely-transcribed DNA involves two separate reactions. In the first strand reaction, complementary DNA (cDNA) is made from a mRNA template using dNTPs and reverse transcriptase. The components are combined with a DNA primer in a reverse transcriptase buffer for an hour at 42°C. The second strand reaction is completed after the reverse transcriptase reaction is complete. cDNA has been generated from the original

single strand-mRNA, and standard PCR (called the "second strand reaction") is initiated. In the two-step RT-PCR process a thermostable DNA polymerase and the upstream and downstream DNA primers are added. Heating the reaction to temperatures above 37°C facilitates binding of DNA primers to the cDNA, and subsequent higher temperatures allow the DNA polymerase to make double-stranded DNA from the cDNA. Heating the reaction to ~95°C melts the two DNA strands apart, enabling other copies of the same primers to bind again at lower temperatures and begin the chain reaction again. After ~30 cycles, millions of copies of the sequence of interest are generated.

Quantitative Real-Time Polymerase Chain Reaction (Q-PCR)

Quantitative real-time polymerase chain reaction (Q-PCR) is also based on polymerase chain reaction where the process enables both detection and quantification of a specific sequence in a DNA sample. Quantification can be as absolute copies of DNA or as a relative amount when normalized to DNA input or additional normalizing genes. The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is quantified as it accumulates in the reaction in *real time* after each amplification cycle. Two common methods of quantification are: (1) the use of fluorescent dyes that intercalate with double-stranded DNA, and (2) modified DNA oligo-nucleotide probes that fluoresce when hybridized with a complementary DNA. It is common to combine real-time polymerase chain reaction with reverse transcription to quantify mRNA in cells.

Cells in all organisms regulate gene expression and turnover of mRNA gene transcripts, and the number of copies of an mRNA transcript of a gene in a cell or tissue is determined by the rates of its expression and degradation. Northern blotting is often used to estimate the expression level of a gene by visualizing the abundance of its mRNA transcript in a sample. In this method, purified RNA is separated by agarose gel electrophoresis, transferred to a solid matrix (such as a nylon membrane), and probed with a specific DNA probe that is complementary to the gene of interest. Although this technique is still used to assess gene expression, it requires relatively large amounts of RNA and provides only qualitative or semi-quantitative information of mRNA levels.

In order to robustly detect and quantify gene expression from small amounts of RNA, amplification of the gene transcript is necessary. The polymerase chain reaction is a common method for amplifying DNA; for mRNA-based PCR the RNA sample is first reverse transcribed to cDNA with reverse transcriptase. Development of PCR technologies based on reverse transcription permits measurement of DNA amplification during PCR in real time, i.e., the amplified product is measured at each PCR cycle. The data thus generated can be analyzed by computer software to calculate relative gene expression in several samples, or mRNA copy number. Real-time PCR can also be applied to the detection and quantification of DNA in samples to determine the presence and abundance of a particular DNA sequence in these samples.

Aim of Study

The objective of this study was to identify all major lipid classes produced by *P. endodontalis* and to determine the potential biologic activity of these ceramide lipids on cells responsible for bone turnover and the modification of immune response when these lipids are present within the necrotic root canal system in order to provide a possible therapeutic approach in the field of endodontic research and clinical practice. The specific aims for this study are:

Specific Aim 1: To identify all major lipid classes produced by *P. endodontalis* and determine whether *P. endodontalis* produces analogous lipids to *P. gingivalis*.

Specific Aim 2: To investigate the potential biologic activity on osteoblast differentiation and mineralization deposition capability and osteoclast differentiation and maturation.

Specific Aim 3: To investigate the potential effect of these lipids on monocyte cytokine production using ELISA analysis.

Materials and Methods

Preparation of *P. endodontalis* Lipids

      P. endodontalis (ATCC 35406, type strain) was grown in batch suspension culture under anaerobic conditions and growth of Gram-negative rods/cocci was verified using Gram stain. *P. endodontalis* was grown under in basal (peptone, trypticase and yeast extract, [BBL] medium supplemented with hemin and menadione (Sigma Co., St Louis) and brain heart infusion as previously described *(Nichols et al., 2004; Nichols et al.*, 2006). The suspension cultures were grown for five days in an anaerobic chamber flushed with N_2 (80%), CO_2 (10%) and H_2 (10%) at 37°C and the bacteria were harvested by centrifugation (3000 x g x 20 min). The bacterial pellets were lyophilized and stored at 20°C until extraction. A sample of *P. endodontalis* pellet was extracted using a modification of the phospholipid extraction procedure of Bligh and Dyer (Bligh *et al.*, 1959) and Garbus *(Garbus et al.*, 1968). *P. endodontalis* lipid extract was fractionated by high-performance liquid chromatography (HPLC) using a semipreparative HPLC column (1 by 25 cm silica gel, 5 µm, Supelco Inc, Bellefonte, PA) eluted isocratically with hexane-isopropanol-water (6:8:0.75, vol/vol/vol: Solvent A) (Nichols et al., 2004).   Replicate fractionations were pooled by fraction number and lipid recovery determined for each fraction as previously described. GC-MS and electrospray-MS analysis (see below)

demonstrated the variety of complex lipids. Once the major lipid fractions were purified to highly enriched fractions, verification of purity was demonstrated by electrospray-MS and GC-MS techniques as previously described.

Electrospray (ESI) MS/MS analysis of dihydroceramide lipids

ESI-MS analysis of lipid fractions was accomplished using a Micromass Quattro II mass spectrometer system (Nichols *et al.*, 2004; Nichols *et al.*, 2006)). The *P. endodontalis* HPLC lipid fractions were dissolved in hexane: isopropanol (6:8, v/v, elution solvent) and injected at a maximum concentration of 100 µg/ml. Lipid samples (10 µl) were infused at a flow rate of 50 µl/min. For electrospray positive ion analyses, the desolvation and inlet block temperatures were 100°C and 150°C, respectively, and the transcapillary potential was 3500 volts. For electrospray negative ion analyses, the desolvation and inlet block temperatures were 80°C and 100°C, respectively, and the transcapillary potential was 3000 volts. The cone voltage is usually 30 volts and the mass acquisition range was 0-2000 amu for initial electrospray MS analyses. MS/MS analysis used a collision energy of between 28 and 30 volts, and argon will be introduced at a pressure of 10^{-2} to 10^{-4} torr. The gas and collision energies were adjusted to minimize parent ion recoveries and maximize daughter ion recoveries. These conditions were used for both positive and negative ion electrospray MS/MS analyses.

Preparation of Osteoclasts

Osteoclast-like cells (OCL) were differentiated from RAW 264.7 cells, a mouse hematopoietic cell line (American Type Culture collection, Rockville, MD). For morphological examination, RAW 264.7 cells were plated at a density of 20,000 cells/well in 24 well plates in alpha modified Eagle medium with 10% fetal bovine serum (Invitrogen, Carlsbad, California). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Assessment of Osteoclast Maturation and Bone-Resorptive Characteristics Induced by Lipid Fractions

Osteoclast Formation- Cultures of RAW 264.7 derived osteoclasts were inoculated with various concentrations of RANKL, RANKL + OPG, and phosphorylated dihydroceramides alone or with OPG. At 1, 2, 3 and 5 days after treatment, cultures were fixed, permeabilized and stained with Texas-red tagged phalloidin (Sigma) to stain microfilaments. The general organization of the actin cytoskeleton was characterized by fluorescence microscopy. A blinded observer determined the number of transduced osteoclasts defined as osteoclasts exhibiting red fluorescence labeling. The observer next determined the number of actin rings, defined as a continuous ring of podosomes. Finally the osteoclasts were stained colorometrically for tartrate resistant acid phosphatase (TRAP) activity (a marker for osteoclasts) and the total number of TRAP positive cells were determined.

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TRAP Assay

Tartrate resistant acid phosphatase (TRAP) is a marker enzyme specific for osteoclasts. At 96 h of culture, cells were fixed with 2% paraformaldehyde, washed with phosphate buffered saline, and treated for 20 minutes with 0.2% Triton X-100 solution to permeabilize cell membranes. Cytochemical staining of tartrate-resistant acid phosphatase (TRAP)-positive cells was performed as described previously (Holliday *et al.,* 2003). TRAP-positive cells appeared dark red. Only TRAP-positive cells with more than 3 nuclei were counted. The values were expressed as the mean ± SE of triplicate cultures.

RNA Extraction, Quantification, and Reverse Transcription

Total RNA was extracted using TRIZOL reagent (Invitrogen) and phenol/chloroform according to manufacturer's instructions. RNA was dissolved in Tris-EDTA (TE), $_P$H 7.4 and the concentration of RNA was determined by measuring the spectrophotometric absorbance at 260 nm. The concentration of extracted RNA was 0.5 µg/µl-1 µg/µl. RNA was treated with DNAse I (Invitrogen) for 15 minutes followed by DNase I inactivation with 25 mM EDTA at 65°C to remove genomic DNA contamination. Reverse transcription was carried out in a 20 µl volume containing about 3 µg of RNA, 1µl of 50 ng/µl random hexamers and 1 µl annealing buffer, 10 µl 2X first-strand reaction mix and 2 µl superscript

III/RNase OUT enzyme mix (Invitrogen) at 25°C for 10 minutes and then at 50°C for 50 minutes.

Quantitative Real-time RT-PCR

Taqman real-time PCR was performed from 1 µl of cDNA using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) with 100-nM primers and a 50-nM probe. The Taqman Real-time RT-PCR was performed on a Taqman ABI 7500 sequence Detection System (Applied Biosystems). Unlabeled specific primers and the TaqMan MGB probes (6-FAM dye-labeled) for detecting the mouse TRAP gene (Assay ID: Mm00475698 m1); calcitonin receptor gene (Assay ID: Mm00432271 m1) and cathepsin K gene (Assay ID: Mm00484036 m1) were used. A Taqman eukaryotic 18S endogenous control kit was used for housekeeping gene control. Cycling conditions were: An initial hold of 2 minutes at 50°C and 10 minutes at 95°C, then the samples were cycled 40 times at 95°C for 15 seconds and 60°C for 1 minute. Each sample was assayed in triplicate.

Traditional PCR used to analyze the amount of PCR product at the end of the reaction does not truly represent the initial amount of starting material whereas real-time PCR monitors the amount of amplicon in the reaction as it is produced during each PCR cycle. As a result, real-time PCR methodology provides fast, precise, and accurate results by monitoring the amplification of products during the reaction and allows quantification of rare transcripts and small changes in gene expression. The Taqman technique is most widely used

for real-time PCR detection techniques. It uses the 5'-3' exonuclease activity of Taq DNA polymerase to cleave a dual-labeled probe annealed to the target sequence during PCR amplification. The probe, a sequence complementary to the mRNA located between the forward and reverse primers, contains both a fluorescent reporter dye at the 5'-end and a quencher dye at the 3'-end. The fluorescent emission activity of the reporter dye is neutralized by a quenching dye when the TaqMan probe is hybridized to its target sequence. During PCR amplification, Taq DNA polymerase cleaves the TaqMan probe into fragments through its 5'-3' endonucleolytic activity. Thus the reporter dye is separated from the quenching dye, resulting in an increase in fluorescence that is directly proportional to the amplification of the molecule.   Applied Biosystem has commercially available fluorescence-labeled primers and probes for mouse osteoclast-specific genes and endogenous control gene 18S ribosomal RNA. Real-time PCR is currently considered the gold standard for quantitative measurement of mRNA, having both high sensitivity and specificity.

Preparation and Treatment of Mouse Primary Calvarial Osteoblasts

Mouse calvaria harvested from 5-7 day old wild-type and toll-like receptor-2 knockout mice were digested with collagenase and trypsin. Calvarial osteoblasts were plated at a cell density of $1.5 \times 10^4/cm^2$. For the first week, the cells were differentiated in Dulbecco's minimal essential medium (DMEM). Differentiation in the second and third weeks took place in an α-MEM which contained ascorbic acid and β-glycerol phosphate.   Treatment groups were

divided into vehicle control, 1.2 μg/ml of *P. endodontalis* lipid extract and 1.2 μg/ml of *P. gingivalis* lipid extract in the form of sonicated micelles. Osteoblast differentiation was evaluated using a stain which selected for alkaline phosphatase production as well as Von Kossa staining in order to visualize mineralization deposition capability.

Preparation and Evaluation of Monocyte Cytokine Production

Monocyte Raw 264.7 cells were plated at a density of 20,000 cells/well in 24 well plates with α-MEM with 10% fetal bovine serum. The cells were treated in three separate groups consisting of vehicle control, 10 μg/ml of *P. endodontalis* lipid extract or 10 μg/ml of *P. gingivalis* lipid extract in the form of sonicated micelles. At 72 hours, media was collected from all three treatment groups and analyzed for TNF-α production via ELISA analysis.

ELISA

Concentrations of TNF-α in culture supernatants were determined by ELISA in triplicate with commercial ELISA Duo systems (R&D systems), according to the respective manufacturer's instructions. For each sample and assay, the means of the triplicate measurements were calculated.

Statistical Analysis

Statistical tests included one factor ANOVA comparing prostaglandin secretion between culture treatment groups and the Fisher PLSD or Scheffe F-tests for significant differences between treatment categories. Quantitative data were obtained as actin ring formation and TRAP-positive multinucleated cell number. Tabulations of experimental and control groups were compared by Student's t-test. A p value of less than 0.05 was considered significant for any statistical comparisons.

Results

Analysis and Comparison of *P. endodontalis* Lipids to *P. gingivalis*

Identification of lipid fractions using gas chromatography-mass spectrometry revealed that *P. endodontalis* inherently produced analogous complex ceramide lipids when compared to the lipids produced by *P. gingivalis*, as seen in Figure 10. Phosphoethanolamine dihydroceramides and phosphatidylethanolamine phospholipids of *P. endodontalis* were identical in mass and had similar retention times on HPLC separation to the same lipid classes previously shown to be recovered from *P. gingivalis*. Other unidentified complex lipids of *P. gingivalis* were also recovered in *P. endodontalis*; however, additional work is required to show that these lipids are identical between the two species.
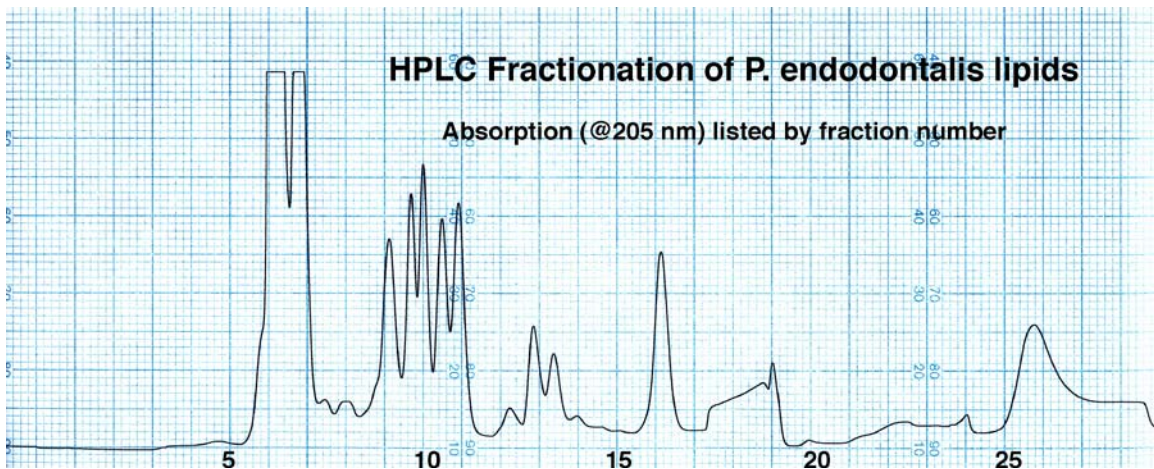

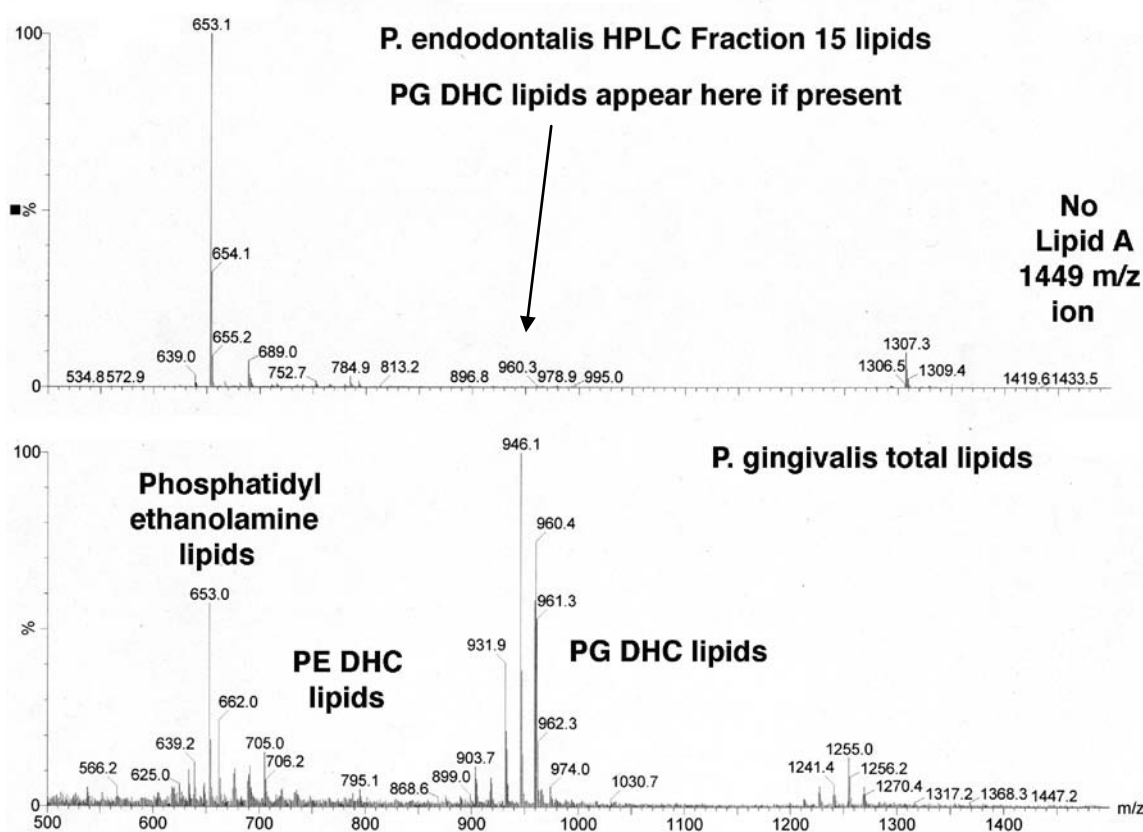Figure 9: HPLC fractionation of P. endodontalis total lipid extract listed by fraction number.

Figure 10: Comparison of total lipids produced by *P. endodontalis* and *P. gingivalis*

However, an important finding during HPLC and electrospray-MS identification of lipids in *P. endodontalis* was the absence of phosphoglycerol dihydroceramide (PG-DHC) production by *P. endodontalis*. Production of *P. gingivalis* PG-DHC can be seen at the bottom of Figure 4 with representative ion peaks of 946.1, 960.4 and 961.3 m/z. Of note, no contamination of lipid A from lipopolysaccharide was observed in any of the lipid samples that were recovered from *P. endodontalis*. The representative molecular ion peak for lipid A *P. gingivalis* is 1449 m/z and this ion was not observed in any the lipid fractions recovered from *P. endodontalis*. This confirmed that phospholipid fractions of *P.endodontalis* were free of lipid A for all biological activity tests.

P. endodontalis Lipids Inhibit Osteoblast Differentiation and Mineralization Deposition Capability _in vitro_

1. Wild-Type Cells:

Evaluation of osteoblast maturation and mineralization deposition capability was performed using biologic stains selective for alkaline phosphatase (an enzyme produced by mature osteoblast cells and important in bone deposition activity) and the production and formation of mineralization nodules. The stain used for the latter activity is known as the Von Kossa stain. Figure 11a-f displays the visual decrease in alkaline phosphatase activity as well as a diminished number of demineralized nodules formed by osteoblasts treated with 1.2µg/ml of _P. endodontalis_ and _P. gingivalis_ total lipid extracts.
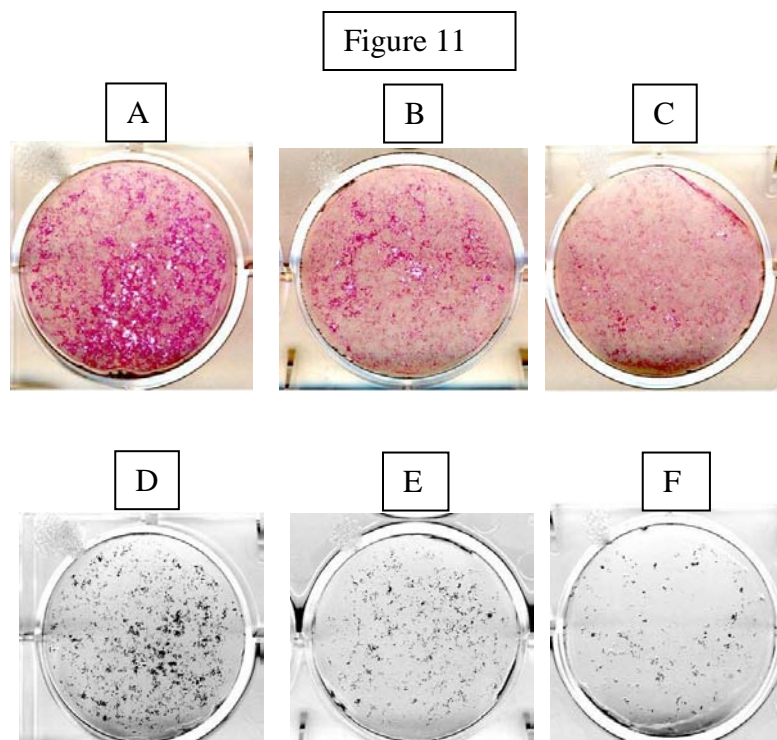

Figure 11

2. Toll-Like Receptor 2 Knock-Out Osteoblasts:

TLR-2 deficient mice were also used to evaluate the potential role of TLR-2 in mediating the effects seen in osteoblast mineralization deposition capability when exposed to total lipid extracts isolated from *P. endodontalis* or *P. gingivalis.* Results indicate a direct relationship between osteoblasts' recognition of bacterial phospholipids and the subsequent decrease in mineralization deposition capability seen by the osteoblast cells. Figure 12 below shows the results of Von Kossa staining of wild-type and TLR-2 knock-out mouse calvarial osteoblasts.
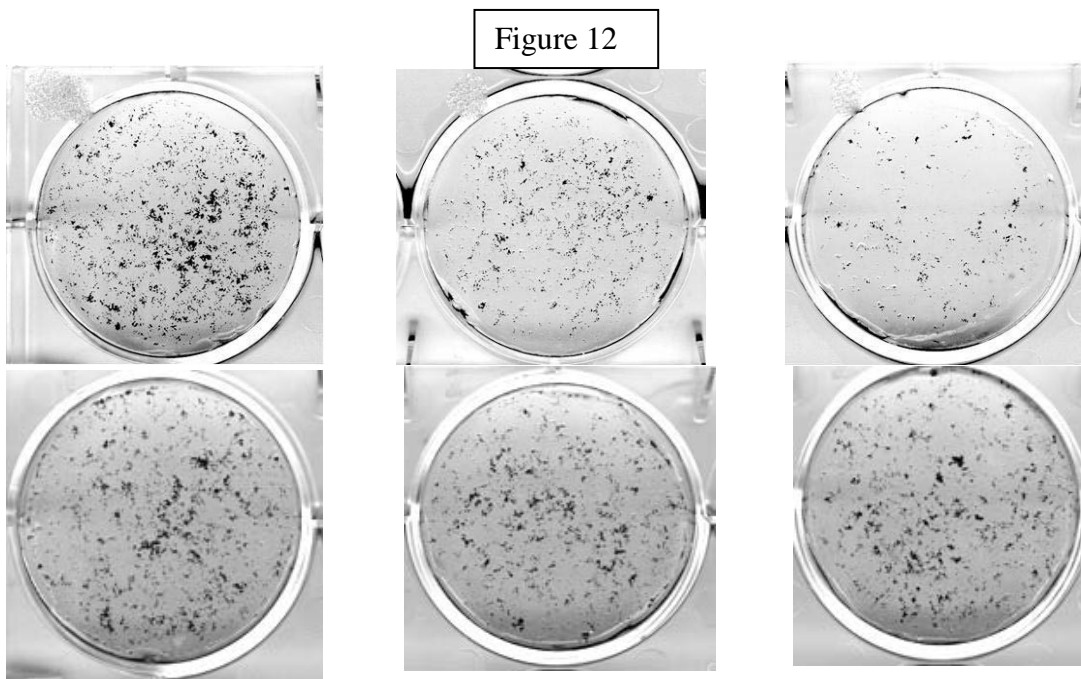

Figure 12

Figure 12: TLR2-KO osteoblasts treated with total lipid extracts from *P. endodontalis* and *P. gingivalis*

When lipid extracts were added to osteoblasts lacking TLR-2, no significant changes were observed in the formation of mineralization nodules by the osteoblast cells when compared to wild-type osteoblasts. This finding may indicate that Toll-like receptor-2 plays a critical role in the induction and maintenance of an osteolytic environment, as seen in apical periodontitis.

P. endodontalis Lipids Stimulate Monocyte Cytokine Production *in vitro*

The next step was to determine whether total lipid extracts from *P. endodontalis* had the ability to stimulate monocyte cytokine production. In the control group, monocytes derived from RAW 264.7 cells were treated with untreated medium. The experimental groups were treated with 10µg/ml of either *P. endodontalis* total lipid extract or *P. gingivalis* total lipid extract for 72h. Utilizing the ELISA analysis technique, we found significant upregulation of TNF-α production by monocytes exposed to lipid extracts of *P. endodontalis* and *P. gingivalis* when compared with the control group.
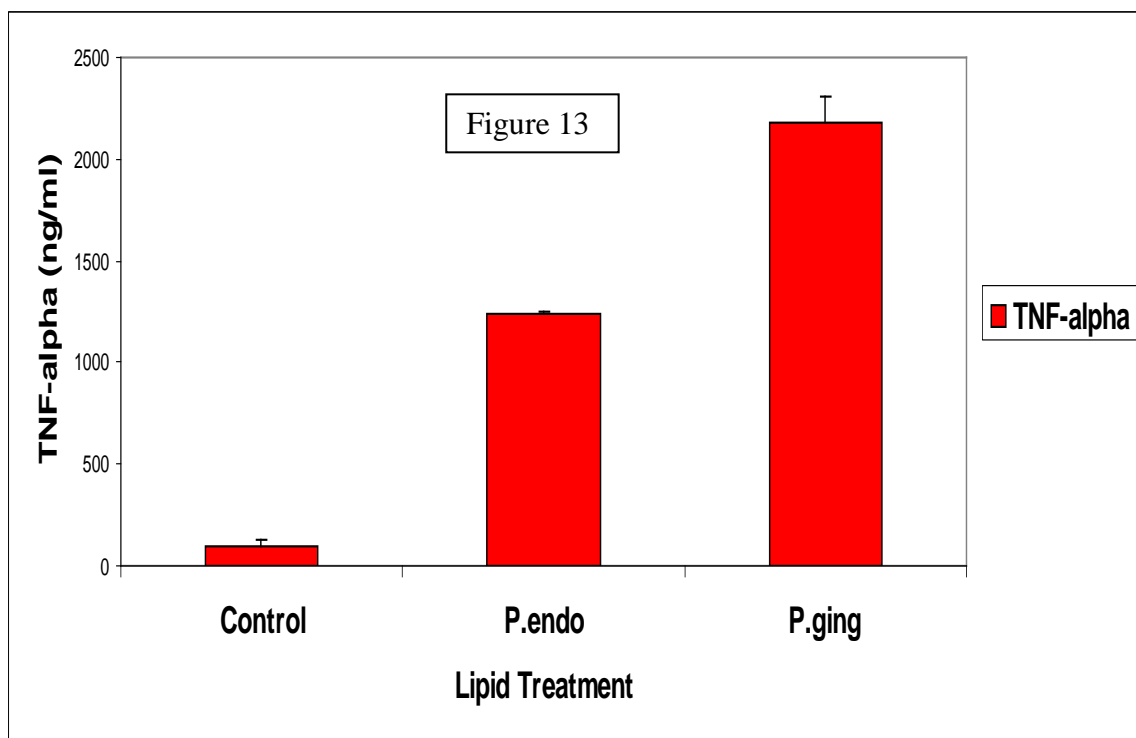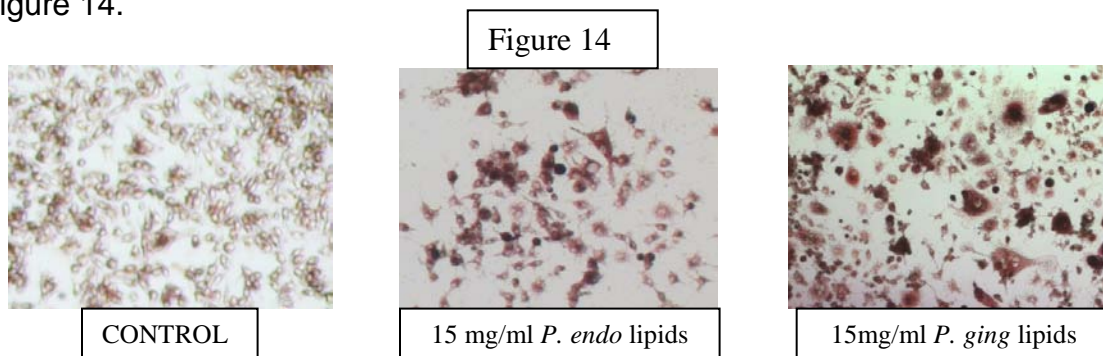

Figure 13

Figure 13 demonstrates the amounts of TNF-α produced by monocytes in each of the experimental groups as well as the control group. Compared with the control group, significantly higher levels of TNF-αincrease (*p= <.05*) in TNF-α production was observed in both of the experimental groups with *P. gingivalis* lipids causing significantly more TNF-α to be produced when compared with *P. endodontalis* lipids.

Presence of *P. endodontalis* Lipids Induces Osteoclast-like Cell Formation

It was our goal to evaluate the potential effect of *P. endodontalis* total lipid extract on inducing osteoclast cell differentiation and maturation. Again, monocytes were plated and treated with control medium, *P. endodontalis* total lipids or *P. gingivalis* total lipids for experimental groups. After 7 days, TRAP+ cells in each were counted in triplicate under magnification. Results are shown in Figure 14.
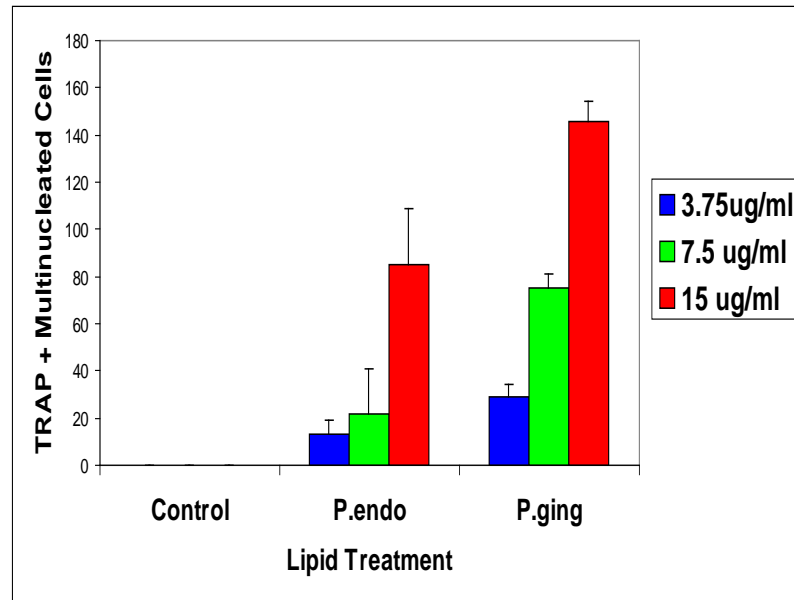

Figure 14

CONTROL | 15 mg/ml *P. endo* lipids | 15mg/ml *P. ging* lipids

Figure 14: Dose-dependent TRAP-positive osteoclast formation occurs in the presence of P. endodontalis and P. gingivalis total lipid extracts

The results demonstrated a dose-dependent increase in TRAP-positive osteoclast cells when exposed to varying concentrations of *P. endodontalis* and *P. gingivalis* lipid extracts. These changes were statistically significant (*p= <.05*) when compared with the control group.

P. endodontalis Lipids Up-regulate Osteoclast-Specific Genes Involved in Bone Resorption

RNA isolated from the osteoclast cells was used to evaluate any upregulation of osteoclast-specific genes that are involved in bone resorption, namely TRAP, cathepsin K and NFATc1. Quantitative real-time RT-PCR was utilized and the results are shown in Figure 15 below.
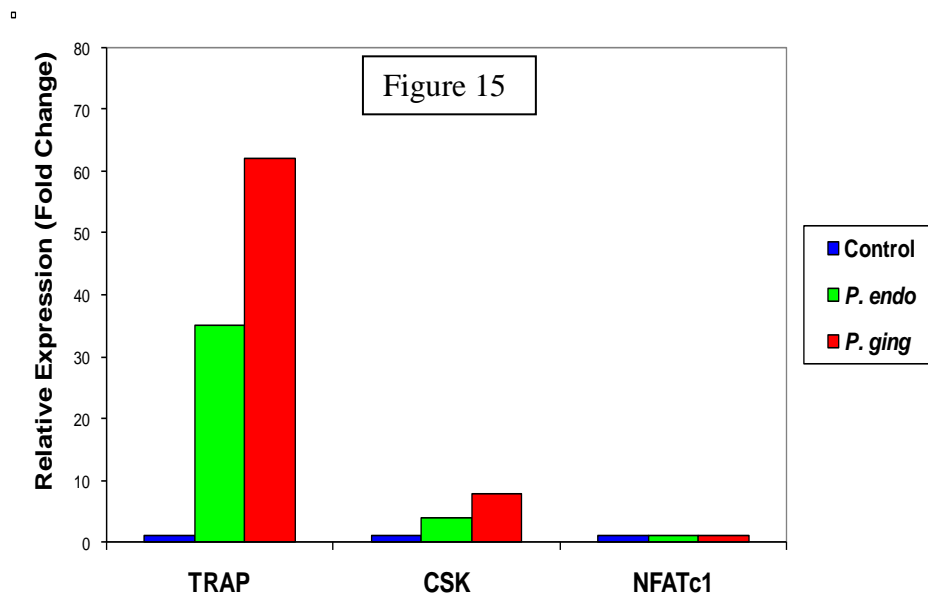
Figure 14: Osteoclast-specific gene expression in the presence of *P. endodontalis* and *P. gingivalis* total lipid extracts.

Significant increases ($p= <.05$) in TRAP and cathepsin K gene expression were observed in osteoclasts exposed to total lipid extracts of *P. endodontalis* or *P. gingivalis*. However, there was no evidence of NFATc1 upregulation seen in either of the experimental groups.

P. endodontalis Lipid Fractions Stimulates Monocyte TNF-α Production

As mentioned previously, monocytes were prepared but this time inoculated with 4 lipid fraction-groups (see Figure 16a, each group containing a high quantity of a specific dihydroceramide lipid). Monocytes were also plated with RANKL, phosphoglycerol dihydroceramide recovered from *P. gingivalis* as well as total lipid extracts from *P. endodontalis* and *P. gingivalis*. An untreated group served as the negative control. Measurements of TNF-α production in each group are displayed in Figure 16b below.

Lipid Fraction Group 1	*P. endo* lipids from fractions 12-17
Lipid Fraction Group 2	*P. endo* lipids from fractions 23-24
Lipid Fraction Group 3	*P. endo* lipids from fractions 25-26
Lipid Fraction Group 4	*P. endo* lipids from fractions 26-27

<u>Figure 16a</u>: Table describing the contents of each *P. endodontalis* lipid fraction group.
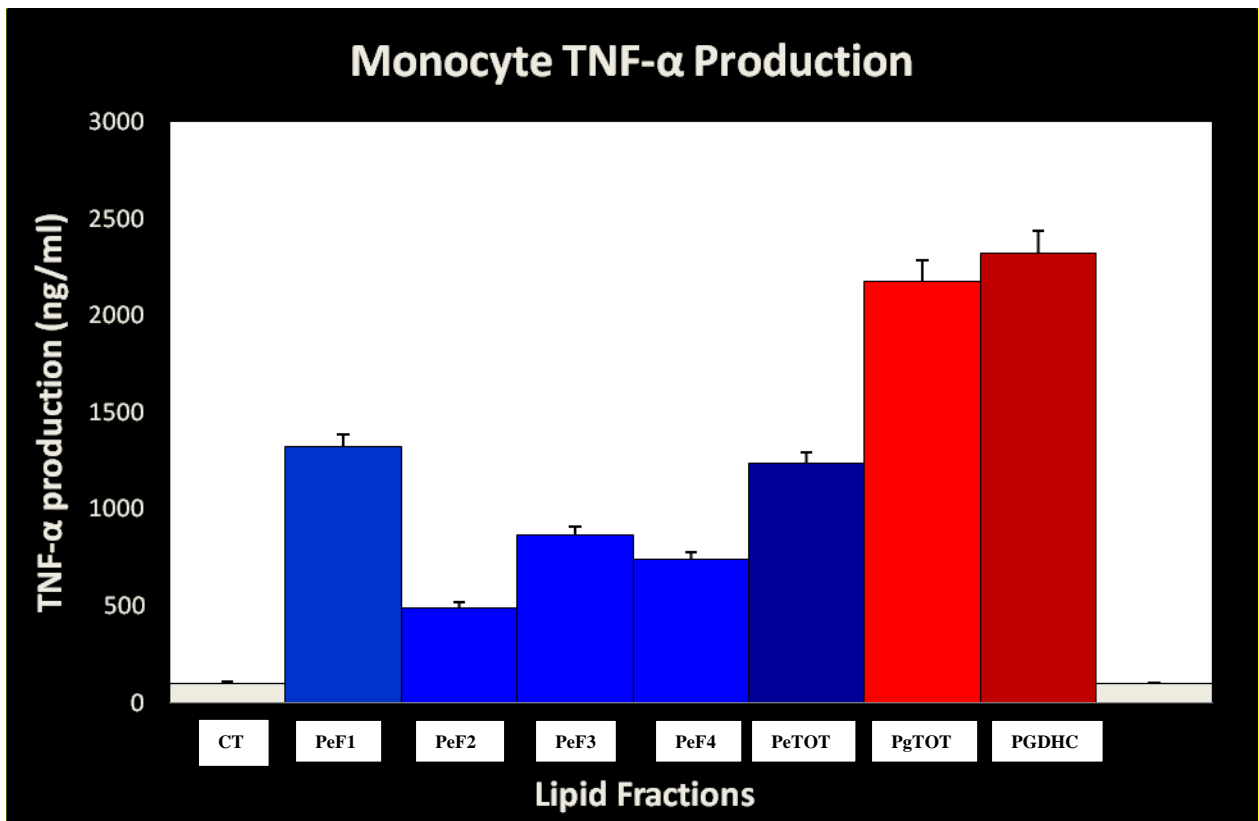
<u>Figure 16b</u>: Graph demonstrating the various levels of TNF-a production by monocytes when exposed to specific lipid fractions from *P. endodontalis*.

Results demonstrated that group 1 (which contained lipid fractions 12-17) showed the significant increase in monocyte TNF-a production compared to all other fraction groups. These measurements were compared to *P. gingivalis* phosphoglycerol dihydroceramide and total lipid extracts as seen in the red bars on the right side of the graph. Lipid fractions 12-17 contained high amounts of a specific lipid with an assigned peak of 653.1 m/z negative ions (see Figure 17). The structure of this lipid has yet to be determined using nuclear magnetic resonance and is a primary goal in future experiments.

Figure 17: GC-MS analysis of lipid fraction 13 from *P. endodontalis* displaying lipid peak 653.0

Discussion
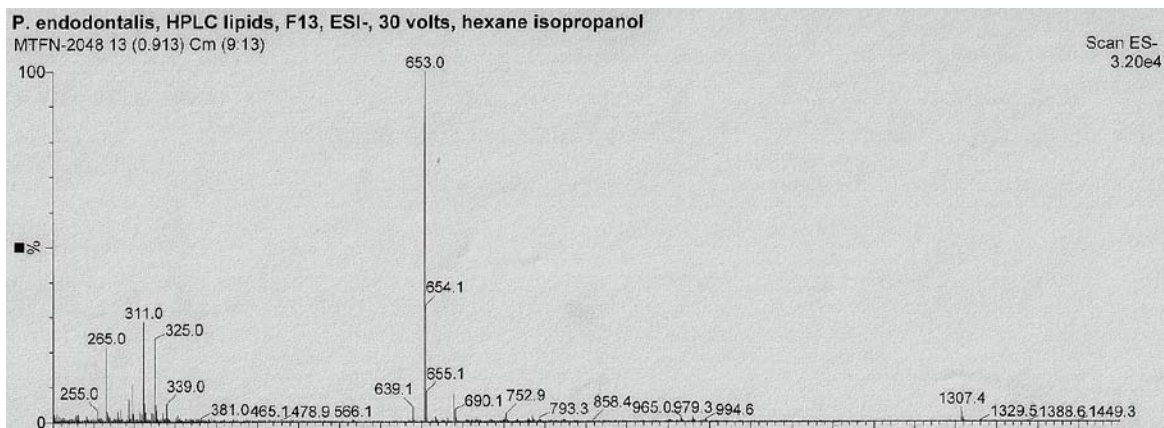

The presence of Gram-negative, black pigmented rods in the root canal systems of necrotic teeth has been well established in the endodontic literature (Sundqvist, 1976; Moller, 1981). Of these, *P. endodontalis* has been identified in numerous studies as a predominant species harbored within an infected root canal space (Sundqvist, 1979; van Winkelhoff *et al.*, 1985. Siquiera *et al.*, 2002). However, little is known about how and if this microorganism plays a significant role in establishing and maintaining an environment which favors bone resorption, as seen in apical periodontitis. Much attention has focused on specific bacterial components and/or by-products that are released by microorganisms into their surrounding environments. Of these, bacterial lipopolysaccharide (LPS) and its' ability to induce immune-mediated, pro-inflammatory events including bone resorption in periapical lesions has been extensively studied. Bacterial lipopolysaccharide (LPS) is an endotoxin and a major component of the outer membrane of Gram-negative bacteria. It is a complex glycolipid composed of a hydrophilic polysaccharide moiety and a hydrophobic domain known as lipid A. Previous studies have demonstrated the mechanisms by which LPS stimulates osteoblasts and surrounding cells to secrete pro-inflammatory cytokines such as TNF-α and IL-6 that are responsible for bone resorptive activity (Nair, 1996). Also, a positive correlation has been reported between the levels of LPS in root canals and the presence of periapical lesions (Dahlen and Bergenholtz, 1980; Schonfeld *et al* 1982).

In our experiments, we chose to study the potential virulence capacity of another crucial component of the cell membrane of specific Gram-negative bacteria, namely phosphorylated dihydroceramide lipids, which make up a sizable percentage of the bacteria's cell membrane in *Bacteroides*. A previous investigation has shown that exposure of these ceramide lipids to gingival fibroblasts significantly potentiated the interleukin-1β (IL-1β)-mediated prostaglandin secretion with marked changes noted in fibroblast morphology (Nichols *et al.*, 2004). The verity that several studies have concluded that *P. endodontalis* is a frequent isolate from necrotic root canal systems spawns the question of whether its presence, alive or dead, has the potential to induce bone destructive and pro-inflammatory events within the host.

Quantification, analysis and comparison of *P.endodontalis* total lipid extract to *P. gingivalis* total lipids was carried out before biological activity was assessed. To date, no studies have described the specific lipid fractions produced by *P. endodontalis*. However, previous experiments have described the structures and biological activities of specific lipid fractions of *P. gingivalis* (Nichols *et al.*, 2004; Nichols *et al.*, 2006). The conclusions were that different lipid fractions from *P. gingivalis* produced varying amounts of pro-inflammatory stimulation to host-derived fibroblasts, namely prostaglandin-E$_2$ secretion. Although the two species of *Porphyromonas* overall produced analogous ceramide lipid fractions when separated using HPLC, an important finding in our experiment was the absence of phosphoglycerol dihydroceramide (PGDHC) lipid production by *P. endodontalis* (see Figures 18 & 19) . It has been previously

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shown that when harvested gingival fibroblasts are treated with isolated PGDHC, a significant morphological change within the fibroblast occurs which may indicate a pro-inflammatory response to foreign material (Nichols *et al.*, 2004). The presence of PGDHC within *P. gingivalis* total lipid extracts may partially contribute to the explanation of why there was a slightly greater effect seen in biologic activity with *P. gingivalis* total lipids compared with *P. endodontalis* total lipids on osteoblast and osteoclast maturation as well as monocyte TNF-α production.
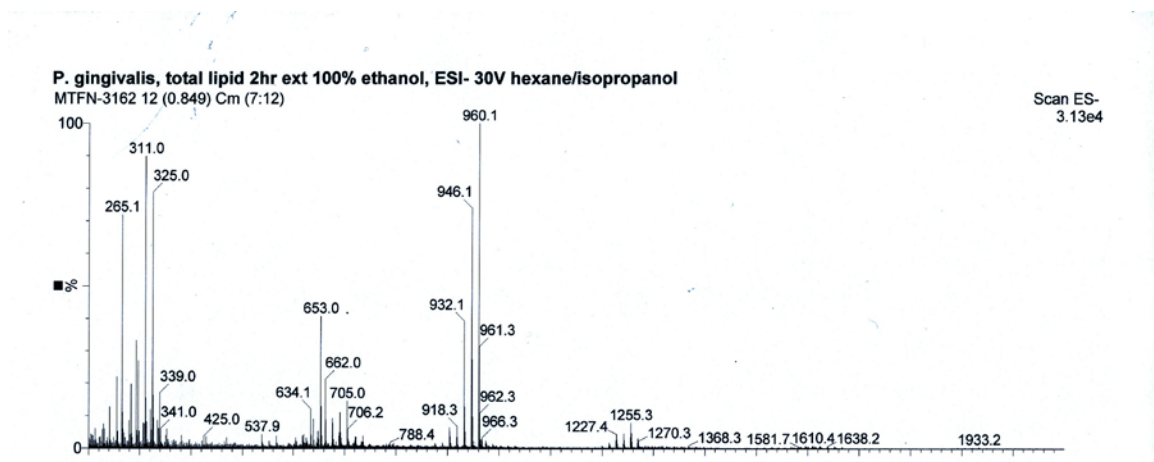

Figure 18: GC-MS analysis of *P. gingivalis* total lipid extract displaying molecular ion peaks of 946.1, 960.1, and 961.3 representative of phosphoglycerol dihydroceramide (PG DHC).
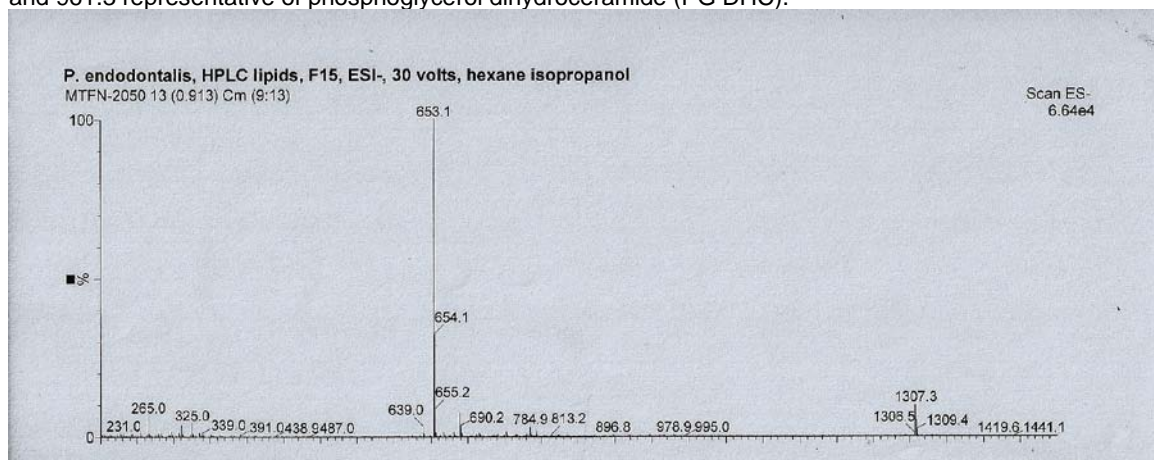

Figure 19: GC-MS analysis of *P. endodontalis* fraction 15 demonstrating no production of PG DHC. Fractions 11-16 are where *P. gingivalis* PG DHC is present in greatest abundance.

Osteoblasts are an integral component of the bone remodeling system. Impairment of osteoblast activity may ultimately result in an environment in favor of bone resorption, with osteoclast activity dominating within periapical lesions of endodontic origin. Our results showed that of isolated ceramide lipids both *P. endodontalis* and *P. gingivalis* caused significant impairment of osteoblast maturation as well as a decrease in their ability to produce and deposit mineralized tissue.

Toll-like receptors are a newly discovered class of trans-membrane receptors found on the surface of immune cells (including osteoblasts and osteoclasts), whose activation has been shown to be critical for the initiation of inflammatory reactions induced by bacterial byproducts. Toll-like receptor 4 (TLR4) is the established receptor for LPS, which is an integral component of the cell wall of Gram-negative bacteria found in the root canals of infected teeth. The relationship between LPS and periapical bone resorption has been well documented in the endodontic literature. More recent findings have shown that LPS can directly stimulate osteoblasts through NF-κβ activation of target genes to express RANKL, which results in the induction of osteoclast formation (Kikuchi *et al.,* 2001). It has been previously demonstrated that LPS can directly induce monocytes to differentiate into mature osteoclasts through a shared mechanism, yet independent from the RANK-RANKL interaction (Jiang *et al.,* 2006). NFATc1 is the most strongly induced transcription factor gene mediated by RANKL whose

presence has been shown to be required during the final stage of osteoclastogenesis. Activation of NFATc1 also results in production of osteoclast-specific genes involved in bone resorption including tartrate-resistant acid phosphatase (TRAP) and Cathepsin K.

A recent study has examined both TLR4 and Toll-like receptor 2 (TLR2) in mediating pro-inflammatory events which result in bone resorption in mice. The study conducted by Burns (2006) examined P. gingivalis effects on bone resorption in Toll-like receptor 2-deficient mice. Cytokine production and bone resorption were measured in both wild-type and TLR2 knockout mice. When *P. gingivalis* was inoculated intraorally into TLR2-deficient mice, an almost complete absence of cytokine production was observed. Also, a marked attenuation of alveolar bone resorption was seen when compared with controls and TLR4-deficient mice (Burns *et al*., 2006). These results led our group to test the isolated ceramide lipids recovered from *P. endodontalis* on TLR2-deficient mouse osteoblasts in order to evaluate the potential role of TLR2 in mediating the effects seen in osteoblast maturation and function. Compared to wild-type osteoblasts, cells from TLR2 knockout animals treated with *P. endodontalis* total lipids, showed no difference in mineral deposit formation in contrast to wild type osteoblasts that showed marked inhibition of mineral deposition with total lipid treatment found in mineralization deposition capability in the osteoblasts which were TLR2-defiicent. Our findings lead us to believe that toll-like receptor 2 plays a significant role mediating osteoblast differentiation mediated by phosphorylated dihydroceramide lipids. To date, no published studies have focused on the exact

mechanism of recognition by immune cells to these complex ceramide lipids. Several studies have examined the effects of *P. gingivalis* LPS and other bacterial byproducts possibly binding to and activating TLR4 and/or TLR2, but whether or not these byproducts contained phospholipid contaminants in addition to their target bacterial components remains to be elucidated (Darveau & Bainbridge 2001; Blair *et al.*, 2009).

Researchers have identified a series of cytokines that are produced by both inflammatory and non-inflammatory host cells that are involved in the bone resorptive process (Stashenko, 1994; Fouad, 1997; Stashenko, 1998). These cytokines include IL-1α, IL-1β, TNF-α, IL-6, and IL-11 (Stashenko, 1994; Manolagas, 1995; Kawashima and Stashenko, 1999). It has also been demonstrated that when an infected root canal is thoroughly cleaned and debrided, rendering it free from bacteria and bacterial byproducts, pulpal and periapical inflammatory responses cease to occur and the damaged tissues heal themselves (Moller *et al,* 1981). Research has confirmed the potent pro-inflammatory activity of bacterial LPS. When LPS is liberated and released into the host tissue where it can initiate an inflammatory response (Rietschel and Brade, 1992a; Barthel *et al.,* 1997) and ultimately result in periapical bone destruction (Stashenko, 1990; Yamasaki *et al.,* 1992). It has been thought that most, if not all, of the pathogenic effects of LPS are not direct, but rather induced indirectly through its interaction with various host cells. LPS stimulates monocytes, macrophages, and various other host cells to produce and release a large number of pro-inflammatory cytokines such as tumor necrosis factor-α

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(TNF-α), interleukin (IL)-1, IL-6, and IL-12 as well as a variety of other small molecules such as lipid mediators, oxygen radicals, and enzymes (Raetz, 1990; Cohen, 2002). These mediators can then exert either a local or systemic response by acting independently, sequentially, synergistically or antagonistically to induce many of the typical effects of LPS (Rietschel and Brade, 1992a). In addition to its pro-inflammatory effects, LPS has been shown to be a potent stimulator of bone resorption by acting on the production and release of cytokines that influence osteoclast differentiation and activation (Ito *et al.,* 1996; Kikuchi *et al.,* 2001; Jiang *et al.,* 2003). With our phospholipids, we observed significant changes in monocyte TNF-α production when compared with untreated controls. Figure 13 demonstrates the difference in TNF-α production by monocytes when exposed to *P. endodontalis* and *P. gingivalis* total lipid extracts. Using these results, we can only speculate that the presence of these bacterial phospholipid dihydroceramides maintains an environment which favors bone resorptive activity and persistence of chronic inflammation within the periradicular area of a necrotic tooth.

Osteoclastic bone resorption consists of multiple steps and studies have identified several key elements thought to be important for osteoclast differentiation (osteoclastogenesis) and activation (Teitelbaum and Ross, 2003). Recent findings support the notion that LPS and some inflammatory cytokines such as TNF-α and IL-1 may be directly involved in osteoclast differentiation and activation through a mechanism partially independent from that of the RANKL-RANK interaction (Jimi *et al,* 1999; Kobayashi *et al.,* 2000; Jiang *et al.,* 2006).

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Our findings suggest that phosphorylated dihydroceramide lipids harvested from *P. endodontalis* stimulates osteoclast differentiation and maturation that may partially be independent of the classic RANKL-stimulated osteoclastogenesis pathway. The lack of NFATc1 upregulation in osteoclasts treated with both *P. endodontalis* and *P. gingivalis* total lipids (see figure15) leads us to the possibility that while these cells indeed produce osteoclast-specific genes involved in bone resorption (figures 14,15), they may be induced to mature via a pathway that is not associated with activation of the NFATc1 transcription factor.

Indeed the question still remains of whether these lipids are present within the necrotic root canal system in large enough quantities to produce an inflammatory response. Future studies will focus on quantifying the amounts of these lipids within the root canal space as well as within periapical lesions of endodontic origin. Lipid-contaminated canal walls could pose a threat in that if left behind after root canal treatment, it is possible these lipids are able to maintain a low level of inflammation in the periradicular area, thus decreasing the overall chance of periapical healing. Sodium hypochlorite, the most commonly used irrigant in endodontics, readily acts on unsaturated fatty acids via hydrolysis with addition of chlorine to one of the carbons and a hydroxyl to the other. The result being an inactivated, non-virulent bacterial byproduct. However, the lipids isolated from *P. gingivalis* contain only saturated fatty acids which contain no double-bonds for the hypochlorous acid to act upon. This is a crucial fact to keep in mind when reviewing the standard irrigation regimen used in endodontics. The exclusive use of sodium hypochlorite in a necrotic tooth could quite possibly

leave behind large quantities of dihydroceramide lipids capable of inducing inflammatory events within the host resulting in non-healing periapical lesions. Other endodontic irrigants and rinses, including chlorhexidine gluconate, may be able to challenge the virulence of dihydroceramide lipids. Chlorhexidine is a cationic molecule that has a high affinity for negatively-charged polar head groups of lipids. This binding and subsequent dissolution of the lipid compound may inactivate its' virulence capacity resulting in a more favorable environment for periapical healing to occur.

Conclusion

In this in vitro study, we were able to successfully isolate and analyze the phosphorylated dihydroceramide lipids produced by *P. endodontalis*. We found a direct pro-inflammatory response when precursor immune cells are treated with varying concentrations of ceramide lipids harvested from *P. endodontalis*. More specifically, we were able to successfully show:

1. *P. endodontalis* produces analogous phosphorylated ceramide lipids as *P. gingivalis* with the exception of phosphoglycerol dihydroceramide which P. endodontalis does not generate.

2. *P. endodontalis* total lipids had an inhibitory effect on osteoblast differentiation and mineralization deposition capability

3. Inhibitory effect on osteoblast mineralization deposition capability was seen through a TLR-2 mediated pathway.

4. *P. endodontalis* total lipids had a stimulatory effect on monocyte TNF-a production compared to untreated controls.

5. Osteoclast cell differentiation and maturation was stimulated with treatment of *P. endodontalis* total lipids with significant increases in enzymes involved with bone resorption.

6. Osteoclast differentiation was observed in absence of NFATc1 transcription factor activation

Although much work still remains in verifying the exact mechanisms of virulence and in determining which lipid fractions induce the most potent inflammatory response in the host, further research in *P. endodontalis* ceramide lipids will no

doubt lead to promising results which will only bring us closer to understanding the complex relationship of bacteria, their byproducts and the destructive host immune response which ultimately results in the generation of apical periodontitis. We can then use these principles in order to apply a more stringent and effective chemo-mechanical debridement technique which will result in a more thoroughly debrided and lipid-free root canal system.

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