6-18-2014

Purification and Biological Activity of Serine Lipids of Porphyromonas endodontalis

Joseph M. Blondin
University of Connecticut School of Medicine and Dentistry, blondin@uchc.edu

Purification and Biological Activity of Serine Lipids of *Porphyromonas endodontalis*

Joseph M. Blondin

D.M.D., University of Connecticut School of Dental Medicine, 2011

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Dental Science

At the

University of Connecticut

2014

APPROVAL PAGE

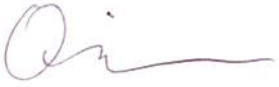
Master of Dental Science Thesis

Purification and Biological Activity of Serine Lipids of *Porphyromonas endodontalis*

Presented by

Joseph M. Blondin, D.M.D.

Major Advisor 
Dr. Frank Nichols

Associate Advisor 
Dr. Qiang Zhu

Associate Advisor 
Dr. Kamran Safavi

University of Connecticut

2014

Table of Contents

Abstract

Porphymonas endodontalis has been shown to populate necrotic root canal systems and may contribute to apical bone loss. Recent work has shown that *P. endodontalis* synthesizes a novel serine lipid class (Lipid 654) similar to that produced by *P. gingivalis.* Objective: The purpose of this study was to isolate *P. endodontalis* Lipid 654 in very high purity and characterize its structure and biological activity in RAW 264.7 cells and HEK293 cells. Methods: *P. endodontalis* (ATCC 35406, type strain) was grown under anaerobic conditions and centrifuged. *P. endodontalis* total lipids were recovered using the Bligh and Dyer phospholipid extraction procedure and were fractionated by semipreparative normal phase HPLC using hexane-isopropanol-water (6:8:0.75, vol/vol/vol). Fractions containing Lipid 654 were identified by mass spectrometric (MS) analysis and were pooled, refractionated using an acidic HPLC solvent, and tested for biological activity. Results: MS analysis revealed that repurified Lipid 654 contained <0.1% contaminating lipids. TNF-α secretion from RAW 264.7 cells was promoted equally by both *P. endodontalis* and *P. gingivalis* Lipid 654 preparations and was significantly attenuated by anti-mouse TLR2 neutralizing antibody. Treatment of HEK293 cells, stably transfected to express TLR2 and an NFκB-dependent secretory alkaline phosphatase reporter, revealed that both *P. endodontalis* and *P. gingivalis* Lipid 654 preparations engage human TLR2 with equal potency. Conclusions: Our results show that *P. endodontalis* Lipid 654 engages TLR2 with stimulation of TNF-α secretion from macrophages and activation of the transcription factor NF-κB, and these responses may contribute to apical bone destruction associated with necrotic pulps. This study was supported by AAE Foundation Research Grant R01 DE021055.

Introduction and Specific Aims

Microorganisms isolated from infected root canals are predominantly Gram-negative and strict anaerobes (1, 2), and *Porphyromonas endodontalis* is frequently the dominant pathogen in necrotic root canals (3, 4). *P. endodontalis* is thought to contribute to immune-activated destruction of periapical tissues through the release of virulence factors including lipopolysaccharide (LPS) (5-8). However, several classes of biologically-active complex lipids, including phosphorylated dihydroceramides and serine lipids, have been shown to be produced by *P. gingivalis* (9-11), an organism that is phylogenetically related to *P. endodontalis*. Of considerable interest, the serine lipids of *P. gingivalis* were recently reported to engage human and mouse Toll-like receptor 2 (TLR2) (11). Furthermore, a survey of oral and intestinal organisms of the phylum *Bacteroidetes* demonstrated that all specimens produce serine lipids of the type recovered in *P. gingivalis* (11). Because of the close phylogenetic relationship between *P. gingivalis* and *P. endodontalis*, the goal of this investigation was to isolate the serine lipid of *P. endodontalis* and determine whether it is structurally identical to the previously identified serine lipid in *P. gingivalis*. Next, this investigation evaluated whether the serine lipid of *P. endodontalis* promotes activation of inflammatory cells, and whether these lipids can be recovered in necrotic root canals of teeth.

Specific Aims:

1. Cultivate *P. endodontalis* and determine whether this organism produces similar phosphorylated dihydroceramide lipids classes as those produced by *P. gingivalis*.

2. Determine whether specific lipid fractions of *P. endodontalis* stimulate TNF-α secretion from RAW 264.7 cells and engage human TLR2 receptors in HEK293 cells, and compare these results to specific lipid fractions of P. *gingivalis*
3. To quantify bacterial lipid levels in lipid extracts from pulpal tissue of extracted teeth

Background Information

Specific interest has been placed in the black-pigmented Gram-negative bacterium *Porphyromonas endodontalis* due to its pathogenic potential and specific involvement in endodontic infections (4). *P. endodontalis* is a Gram-negative, obligatory anaerobic, non spore-forming bacterium and appears as non-motile rods or coccobacilli (12). *P. endodontalis* is a non-fermentative anaerobic bacterium, which requires specific growth factors such as hemin and menadione and is extremely sensitive to oxygen (4). Due to these specific growth requirements, *P. endodontalis* cannot be easily detected by conventional methods and its detection in root canal infections may be underestimated (12).

Although the exact mechanisms of pathogenicity of *P. endodontalis* are not well known, *P. endodontalis* contains several pathogenic factors (13). The end-products of hemoglobin metabolism by *P. endodontalis* consist of n-butyric acid, acetic acid, hydrogen sulfide, and methyl mercaptan, which are all cytotoxic. *P. endodontalis* produces proteases and peptidases, which are able to degrade fibrinogen, fibronectin, collagen type IV, and also cleave angiotensin. A proline aminopeptidase produced by *P. endodontalis* degrades bradykinin and vasopressin. *P. endodontalis* has been found to

elevate MMP-2 production in both human pulp and periodontal ligament (PDL) cell cultures. *P. endodontalis* has been shown to stimulate the vascular network coincident with progression of inflammation through the release of vascular endothelial growth factor (VEGF). In addition, *P. endodontalis* stimulates IL-6 production and IL-10 production, both of which may also contribute to its inflammatory reaction (13).

P. endodontalis, as a Gram-negative bacterium, contains lipopolysaccharide (LPS) in its cell wall, so much of the focus of its pathogenicity has focused on LPS. LPS typically consists of a hydrophobic domain known as lipid A (or endotoxin), a non-repeating "core" oligosaccharide, and a distal polysaccharide (or O-antigen). LPS is detected by a receptor of the innate immune system present on macrophages and endothelial cells. These receptors, called Toll-like Receptors (TLRs), represent a diverse family of molecules that play a critical role in activating the innate immune system in response to pathogens (11). Two of these receptors, Toll-like receptor 4 (TLR4) and Toll-like receptor 2 (TLR2), have been implicated in periodontal inflammation. TLR4 is a membrane-spanning protein that is distantly related to the IL1 receptor. In macrophages, Lipid A activation of TLR4 triggers the biosynthesis of diverse mediators of inflammation, such as TNF-α and IL-1β, and activates the production of co-stimulatory molecules required for the adaptive immune response (14). Although Gram-negative bacteria are generally recognized by TLR4, there is controversy regarding recognition of the Gram-negative bacteria because studies have shown that *P. gingivalis* can signal via TLR2, TLR4, or both (15).

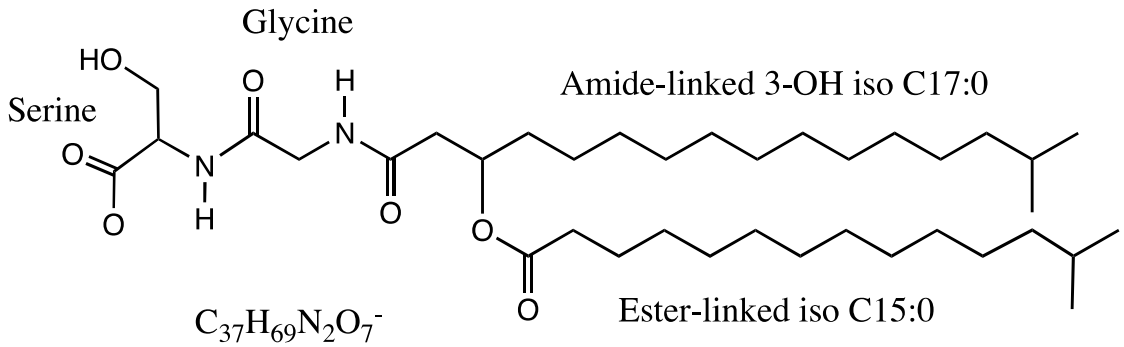
Since *P. gingivalis* is present in the oral cavity, researchers have examined the specific molecules that activate TLR receptors since they play a pivotal role in initiating

immune responses critical to neutralizing microbial threats to the host. *P. gingivalis* has been associated with periodontitis primarily thought TLR2 engagement (16), and one consequence of the pro-inflammatory cytokines produced from TLR2 activation is the damaging effects on alveolar bone, resulting in bone loss as demonstrated by studies using $TLR2^{-/-}$ mice (17). Therefore, LPS from *P. gingivalis* has been purified and examined for activation of the TLR2 receptor. One study found that preparations of LPS from *P. gingivalis* exhibited potent TLR2, rather than TLR4 agonist activity, and to elicit gene expression and cytokine secretion in murine macrophages and transfectants (18). This has been in contrast with other studies that have shown both TLR2 and TLR4 receptor activity by LPS isolated from enterobacteria (19, 20).

Researchers have examined the preparations of LPS species to explain the differences in specific TLR activation. It has been found that many commercial preparations of *E. coli* LPS contain low concentrations of highly bioactive contaminants. Using a method shown to remove endotoxin proteins from LPS preparations, the TLR activity of these endotoxin proteins was examined (21). Activity was compared between cells from wild-type mice and cells transfected with TLR2 and TLR4 receptors. It was found that the unpurified LPS was able to stimulate cells from the wild type mice that express TLR2 and other TLR receptors. Eliminating endotoxin protein from LPS did not influence the ability to stimulate cells from wild-type mice or cells that express TLR4, however re-purification eliminated the ability of LPS to active cells with the TLR2 receptor. Additionally, the potent endotoxin protein contaminants stimulated cell lines with the TLR2 receptor. These results point to the protein contaminants of LPS to be the active agents in stimulating the TLR2 signaling response as opposed to LPS (20).

Using this information, the lipids from *P. gingivalis* have been examined to look for potential contaminants that could trigger the TLR2 receptor. *P. gingivalis* LPS prepared by the commonly used Tri-Reagent method was evaluated for contaminants (22). LPS preparations were examined after additional purification approaches, including the Bligh and Dyer extraction procedure, were applied to remove contaminating phospholipids. It was also shown, using mass spectrometric methods, that up to three successive ethanol washings of the Tri-Reagent-prepared LPS of *P. gingivalis* will recover significant levels of contaminating phosphorylated dihydroceramide lipids. This indicates that LPS purification techniques will only partially remove phosphorylated dihyrdroceramide lipids from LPS, and that LPS purified by the Tri-Reagent method is contaminated with additional serine lipids (22).

Using HPLC together with mass spectrometric/nuclear magnetic resonance spectroscopy, a new serine lipid class of *P. gingivalis*, Lipid 654, has been identified that acts as a ligand for human and mouse TLR2. The structure of this lipid is shown below.



$C_{37}H_{69}N_2O_7^-$
Exact Mass: 653.51
Mol. Wt.: 653.95

Lipid 654

While Lipid 654 shares all structural characteristics with the previously described lipid

class termed “Flavolipin” (23, 24, 25), we have found significant differences between Lipid 654 and Flavolipin in biological activity. Flavolipin was named after the organisms of the *Flavobacterium* genus from which it was originally isolated. According to previous reports, Flavolipin represented approximately 20% of the total cellular lipids of *Flavobacterium meningosepticum* and was capable of acting as a TLR4 ligand (23, 25). In contrast, the results of our study demonstrate that Lipid 654 does not activate via TLR4 but rather functions as ligand for TLR2. Since Lipid 654 acts as a TLR2 ligand but does not appear to activate through engagement of TLR4, it is possible that a lipid contaminant in the Flavolipin preparations accounted for the previously reported TLR4 engagement (23).

An additional important distinction between Flavolipin and Lipid 654 is that while only *Flavobacterium* species were originally reported to produce Flavolipin (23, 24), it is reported that Lipid 654 is produced by more than *Flavobacterium* species. In addition to *P. gingivalis,* analysis of lipid extracts from *Prevotella intermedia, Tannerella forsythia, Capnocytophaga ochracea, C. gingivalis* and *C. sputigena* revealed that each of these oral *Bacteroidetes* produce the Lipid 654 class (data not shown). Selected intestinal *Bacteroidetes* including *Prevotella copri, Parabacteroides merdea, Bacteroides fragilis,* and *B. vulgatis* also produce the Lipid 654 class (data not shown)**.** Our evidence thus far indicates that Lipid 654 is produced by a wide variety of *Bacteroidetes* species and that, unlike Flavolipin, is not produced exclusively by *Flavobacterium* species (23, 25).

Using HEK293 cells stably transfected with TLR2 along with other relevant receptors, Lipid 654 of *P. gingivalis* was shown to activate human cells via TLR2. Lipid

654 contains two fatty acids with one held in ester linkage (see Figure above). The Lipid 654 class will not dissolve in aqueous solvents unless it is sonicated to form liposome preparations. A recent report now suggests that *P. endodontalis* is also capable of producing Lipid 654. In the present investigation, we sought to isolate Lipid 654 from *P. endodontalis* in high purity, verify its structure and determine whether it activates human and mouse TLR2.

Activation of TLR2-expressing HEK293 cells does not appear to be mediated by the phosphorylated dihydroceramides or phospholipid preparations of *P. gingivalis*. These other lipid classes of *P. gingivalis* are prominent sphingolipids that do possess biological activity in other cell assay systems. These phosphorylated dihydroceramide lipids are important to consider because of their separation characteristics. With normal phase HPLC fractionation, Lipid 654 of *P. gingivalis* elutes slightly earlier than the phosphoethanolamine dihydroceramide (PE DHC) lipids but together with phosphatidylethanolamine (PEA) lipids. Though these latter two lipid classes do not engage TLR2, their trace but measurable contamination of the Lipid 654 class poses questions as to their potentiation of Lipid 654 biological effects. With the use of state-of-the art HPLC equipment which was not available at the time of our earlier work, the Lipid 654 class can now be isolated largely free of PE DHC lipids.

It is concluded from this evidence that Lipid 654 is a structurally unique TLR2 ligand derived from *P. gingivalis*. In attempting to clarify the role of TLR2 co-receptors in the engagement of Lipid 654, it was observed that neutralizing antibody against either TLR1 or TLR6 partially inhibited HEK cell activation (data not shown), but not to the extent that anti TLR2 antibody inhibited HEK cell responses. In addition, the pattern of

inhibition with the co-receptor antibodies appeared to differ between Lipid 654 and Lipid 430. Further work is needed to verify these observations.

In unpublished studies, we have recently evaluated lipid extracts of human impacted third molars using Multiple Reaction Monitoring MS, specifically quantifying the Lipid 654 class. It was found that these lipid extracts contain minimal amounts of Lipid 654 (data not shown). These results suggest that in human tissues not directly exposed to bacteria, these lipids are not recovered in appreciable levels. In contrast, recent studies revealed that lipid extracts from diseased human gingival tissue, carotid atheroma and serum, demonstrate Lipid 654 (data not shown) in levels exceeding the phosphorylated dihydroceramide lipids of *P. gingivalis* (26). Therefore, future studies will focus on the recovery of 654 and 430 lipids in healthy and diseased human tissues including healthy and necrotic root canals of teeth.

Although many virulence factors of *P. gingivalis* have been proposed to contribute to periodontal bone and tissue destruction (27, 28, 29), various complex lipids of *P. gingivalis* are readily detected in periodontal disease sites without concurrent bacterial invasion or LPS recovery (30, 31). Further work has shown that specific lipid classes that are relatively minor constituents of this organism are responsible for activation of TLR2 and that dihydroceramide lipids are not responsible for these TLR2-mediated biological responses. In a study attempting to quantify the amount, Clark et al. determined that LPS of *P. gingivalis* prepared according to the methods of Yi and Hacket and Caroff contains an average of 3.81% by weight of lipid 654 (11).

These results are consistent with those of other investigators who have reported that *P. gingivalis* colonization of teeth in experimental animals mediates bone loss

through engagement of TLR2 (28, 32, 33). Lipid 654 produced by *P. gingivalis* and other oral *Bacteroidetes* may play a critical role in the development of destructive periodontal disease by promoting bone loss and inhibition of bone formation as previously reported (34). Lipid 654 may also contribute to the development of other chronic inflammatory diseases of humans where these bacterial lipids accumulate in substantial levels due to contributions from microbes of the oral cavity, GI tract, and other anatomical sites. The purpose of this investigation is to determine if Lipid 654 is also important in pulpal disease.

Materials and Methods

Bacterial Culture and Preparation of Lipids

P. endodontalis (ATCC 35406, type strain) and *P. gingivalis* (ATCC 33277, type strain) were grown in batch suspension culture under anaerobic conditions as previously described (9). Bacteria were cultured in Brain Heart Infusion broth under anaerobic conditions and culture purity was confirmed by demonstrating uniform colony formation on blood agar plates. *P. endodontalis* does not grow on blood agar plates when cultured under aerobic conditions. Gram stain of *P. endodontalis* samples revealed Gram-negative pleomorphic rods. *P. endodontalis* and *P. gingivalis* lipids were extracted using the procedure of Bligh and Dyer (35) and Garbus (36). *P. endodontalis* and *P. gingivalis* lipid extracts were fractionated by high-performance liquid chromatography (HPLC) as previously described (9). Replicate HPLC fractionations were pooled and evaluated using electrospray-mass spectrometry (ESI-MS). ESI-MS analysis of lipid fractions was accomplished using an ABSciex QTrap 4000 mass spectrometer system (9, 10). The

fractions with specific lipid components were pooled and tested for biological activity as described below. The fractions containing Lipid 654 were pooled and refractionated using normal phase separation but with the HPLC solvent supplemented with 0.1% acetic acid. Recovery of Lipid 654 and its purity were confirmed using MS analysis as described above.

Preparation and Evaluation of Monocyte Cytokine Production

Monocyte Raw 264.7 cells were plated at a density of 20,000 cells/well and treated with vehicle control, 10 μg/ml of *P. endodontalis* total lipid extract or 10 μg/ml of *P. gingivalis* total lipid extract in the form of sonicated liposomes. After 24 hours, media samples were collected and analyzed for tumor necrosis factor-alpha (TNF-α) production via ELISA analysis (Duo systems, R&D systems). The Lipid 654 fraction of *P. endodontalis* lipids was evaluated in parallel with the total lipid extracts (see below). Cells were also pretreated for one hour with 1 μg/ml of mouse anti-TLR2 antibody before treatment with Lipid 654. Culture supernatants were collected after 24 hours of exposure to Lipid 654.

Human embryonic kidney cells (HEK293 cells), transfected with either human TLR2 or human TLR4 and stably expressing MD-2 and CD14, were purchased from InvivoGen. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM;Gilbco) containing 4.5 g/liter $_{L}$-glucose and 10% fetal bovine serum (FBS). The activities of specific TLR agonists were measured through a colorimetric assay for the secretory embryonic alkaline phosphatase (SEAP), a reporter gene that is linked to NF-$_{K}$B activation. Measurement of SEAP activity using the Quanti-blue substrate

(InvivoGen) after TLR agonist treatment was carried out in a test medium (DMEM, 10% FBS) without antibiotics according to the manufacturer's instruction. NF-$_K$B activation was expressed as a ratio for each stimulus relative to SEAP activity in unstimulated (vehicle control) cells. For *in vitro* testing, all lipid preparations tested were solubilized in a 50% mixture of dimethyl sulfoxide (DMSO)-water (approximately 1.11% DMSO in the final culture medium).

Preparation of teeth for recovery of Lipid 654 in teeth with a vital and necrotic root canal system.

Recovery of Lipid 654 in pulp canals

Extracted teeth included two basic categories of clinical samples: One type included teeth extracted that are confirmed by electric pulp testing to have necrotic pulpal contents and the other type were teeth extracted for reasons other than pulpal problems. The latter type of teeth were fully impacted third molars that were surgically removed by an Oral Surgeon. The teeth samples were collected in compliance with the IRB approved protocol of the University of Connecticut Health Center. The extracted teeth samples were stored frozen until processing samples in the laboratory. The teeth were first cleaned of adherent soft tissue and scrubbed with household bleach. The crown of each tooth was removed using a hand piece and the exposed pulp contents were reamed out with a bur (Peezo Reamer). Each reamer was placed in an individual glass tube and lipids extracted using a chloroform:methanol lipid phospholipid extraction procedure described above. The extracted lipids were analyzed for Lipid 654 using multiple reaction monitoring-mass spectrometry on the ABSciex QTrap 400 instrument. This

mass spectrometric method quantifies the specific bacterial lipids that are thought to contribute to the development of pulpal disease and bone loss.

Statistical Analysis

Statistical tests included one-factor ANOVA comparing differences between culture treatment groups. A Mann-Whitney U test was used to evaluate for significant differences between teeth with a vital pulp and teeth with a necrotic pulp. A p value of less than 0.05 was considered significant for statistical comparisons.

Results

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

Identification of lipid fractions using gas chromatography-mass spectrometry revealed that *P. endodontalis* inherently produced analogous complex serine lipids when compared to the lipids produced by *P. gingivalis* (see Figure 1).

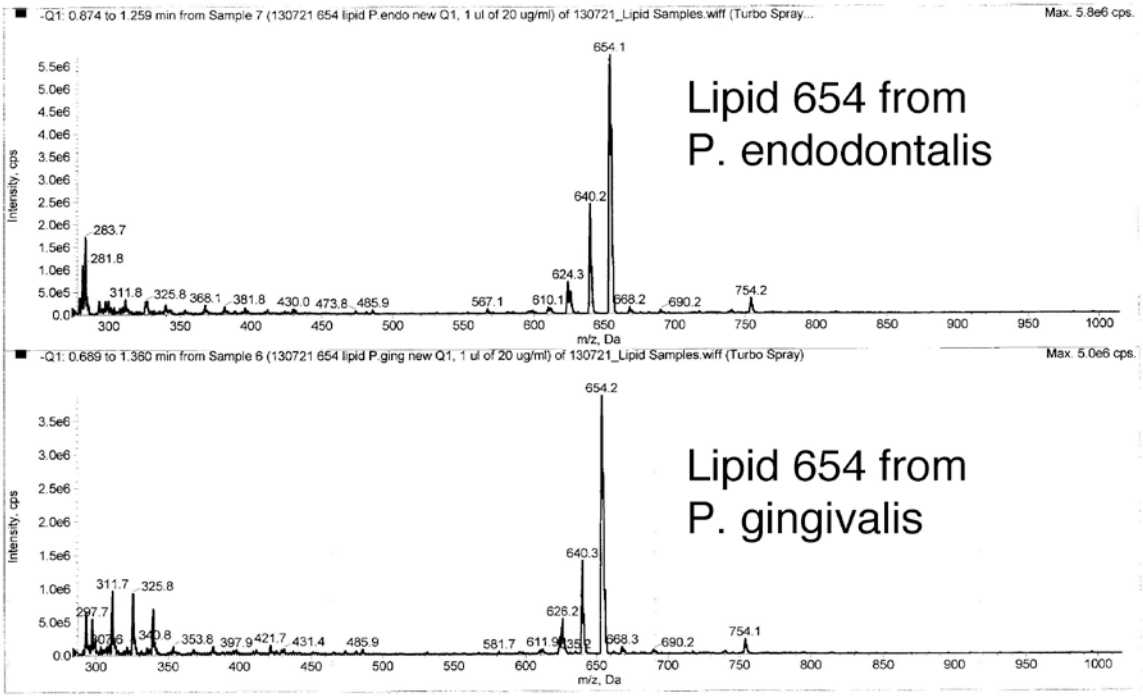


Figure 1: Comparison of representative electrospray mass spectra for pooled Lipid 654 fractions from *P. endodontalis* and *P. gingivalis*. The serine lipids are identified in both bacteria, with Lipid 654 as the most abundant species within this lipid class.

The major species is present with an m/z of 654 negative ion. The serine lipids of *P. endodontalis* were identical in mass and had similar retention times on HPLC separation to the same lipid classes previously recovered from *P. gingivalis* (9). No lipid A, which has an atomic mass around 1400, was observed using electrospray-mass spectrometry.

The negative ion MS/MS analysis of the *m/z* 654 precursor ions revealed the MS/MS spectra shown in Figure 2.

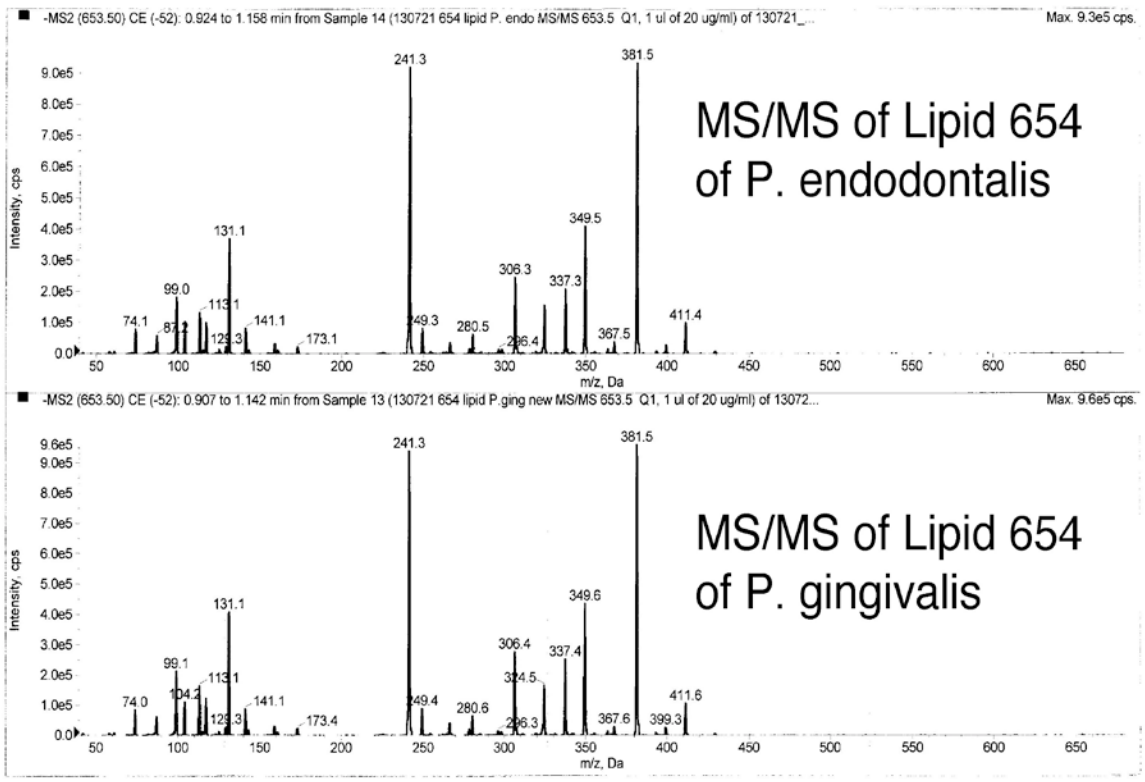


Figure 2: MS/MS profiles of Lipid 654 from *P. endodontalis* and *P. gingivalis*. Identical ion fragments of the 654 precusor ions are identified in fractions from both *P. endodontalis* and *P. gingivalis*.

The ion peaks are the same for Lipid 654 from *P. endodontalis* and *P. gingivalis*, which indicates that the precursor Lipid 654 ion has the same structure. Using this information, the structure of Lipid 654 from *P. endodontalis* is proposed to be the same structure as Lipid 654 from *P. gingivalis* (see Figure 3).

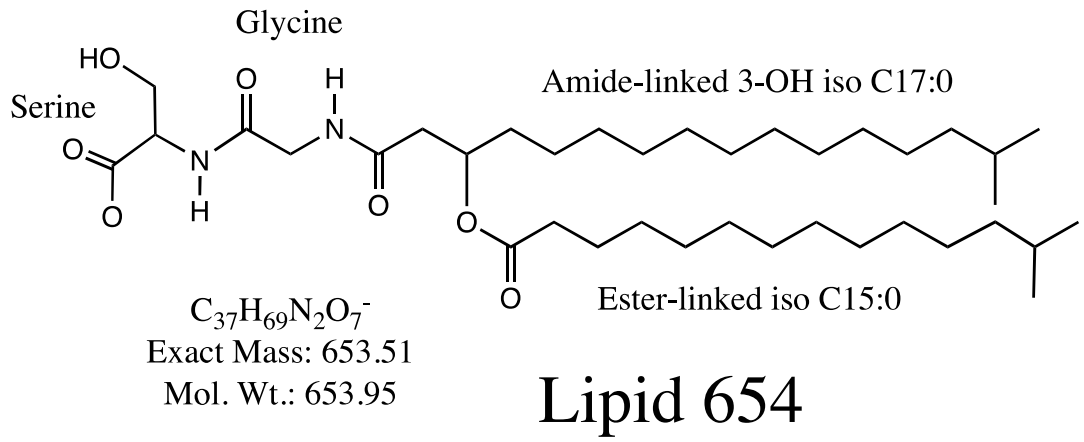


Figure 3: Proposed lipid structure of *P. endodontalis* Lipid 654.

The proposed lipid structure in Figure 3 shows the most abundant species of the Lipid 654 class.

Preparation and Evaluation of Monocyte Cytokine Production

RAW 264.7 cells were treated with Lipid 654 pooled from fractions of *P. endodontalis* lipids that were previously fractionated by HPLC. These pooled fractions were then used to treat RAW 264.7 cells in culture. Monocytes were also treated with total lipid extracts from *P. endodontalis* and *P. gingivalis*. An untreated group served as the negative control, and LPS served as a positive control. Measurements of TNF-α production in each group are displayed in Figure 4 below.

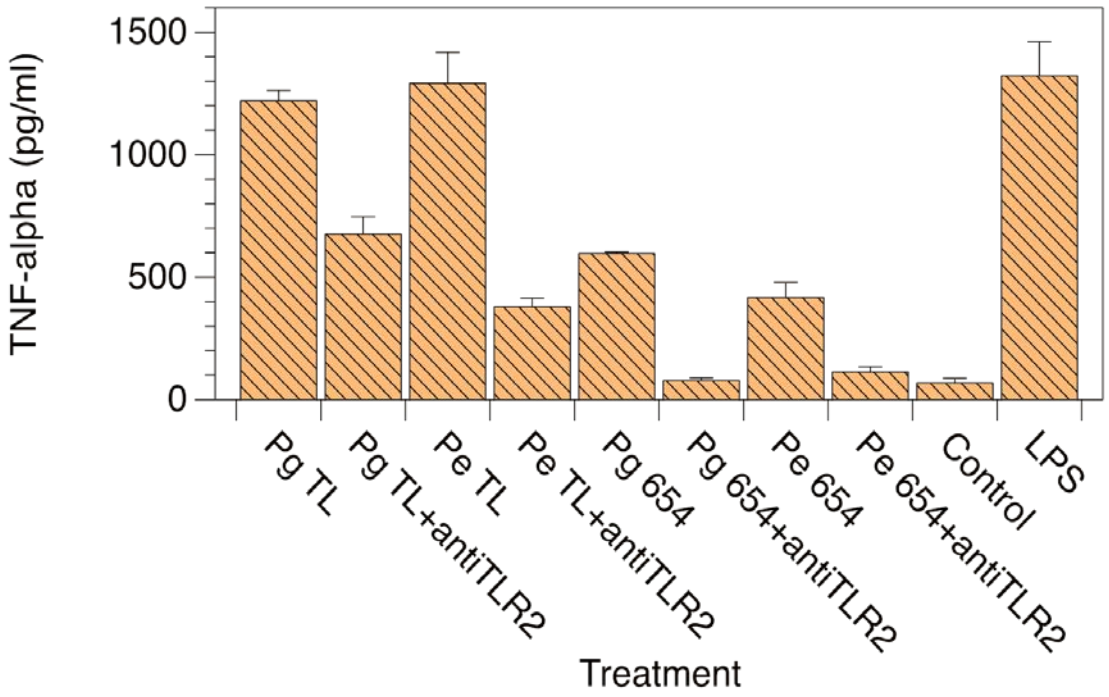


Figure 4: TNF-α secretion from RAW 264.7 cells was promoted by both *P. endodontalis* and *P. gingivalis* total lipid and Lipid 654 preparations. This promotion was significantly attenuated by anti-mouse TLR2 neutralizing antibody. Data are averaged from 3 separate trials.

TNF-α secretion from RAW 264.7 cells was significantly promoted by both *P. endodontalis* and *P. gingivalis* total lipid and Lipid 654 preparations compared to control. Elevated TNF-α secretion was significantly attenuated by anti-mouse TLR2 neutralizing antibody. The data are averaged from 3 separate trials.

We next evaluated the biological activity dose-response characteristics of lipid 654 from *P. endodontalis* and compared these responses with a well-characterized TLR2 agonist as well as Lipid 654 from *P. gingivalis* for their abilities to activate TLR2-expressing HEK293 cells. Compared with the known TLR2 ligand positive control

MMP, Lipid 654 promoted HEK cell activation over the control cells (DMSO-treated cells) (see Figure 5).

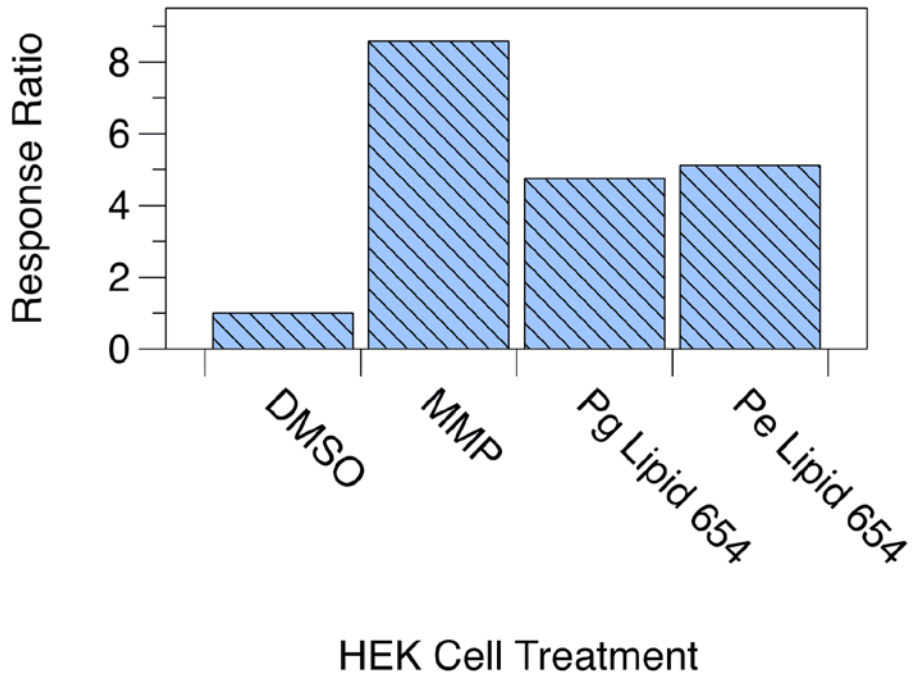


Figure 5: HEK Cell activation by *P. gingivalis* and *P. endodontalis* Lipid 654. HEK cell activation levels by MMP (a known TLR2 ligand), *P. gingivalis* Lipid 654 and *P. endodontalis* Lipid 654 were elevated compared with DMSO vehicle.

MMP (molecular weight of 1,269.82) was used at a concentration of 0.2 μg/ml, or 0.158 μM. Lipid 654 was used at a concentration of 0.7 μg/ml representing 1.066 μM. Results could not be tested for significance as only one trial was performed, but a substantial increase in response ratio was observed.

Recovery of Lipid 654 in teeth with a vital and necrotic root canal system

Ten teeth with vital pulps and ten teeth with necrotic pulps were used to evaluate the amount of Lipid 654 present. Multiple Reaction Monitoring Mass Spectrometry

revealed the presence of Lipid 654 in all samples. Intensity of phosphorylated dihydroceramides and Lipid 430 were also examined and are shown in Figures 6 and 7. The intensity of Lipid 654 is minimal in the tooth with a vital pulp (see Figure 6), while the intensity of Lipid 654 in the tooth with a necrotic pulp is much higher (see Figure 7). The abundance of phosphorylated dihydroceramides and Lipid 430 in teeth with vital pulps and teeth with necrotic pulps are similar, indicating that these lipids are not more abundant in teeth with necrotic pulps and likely are not heavily involved in the inflammatory reaction (see Figure 6 and 7).

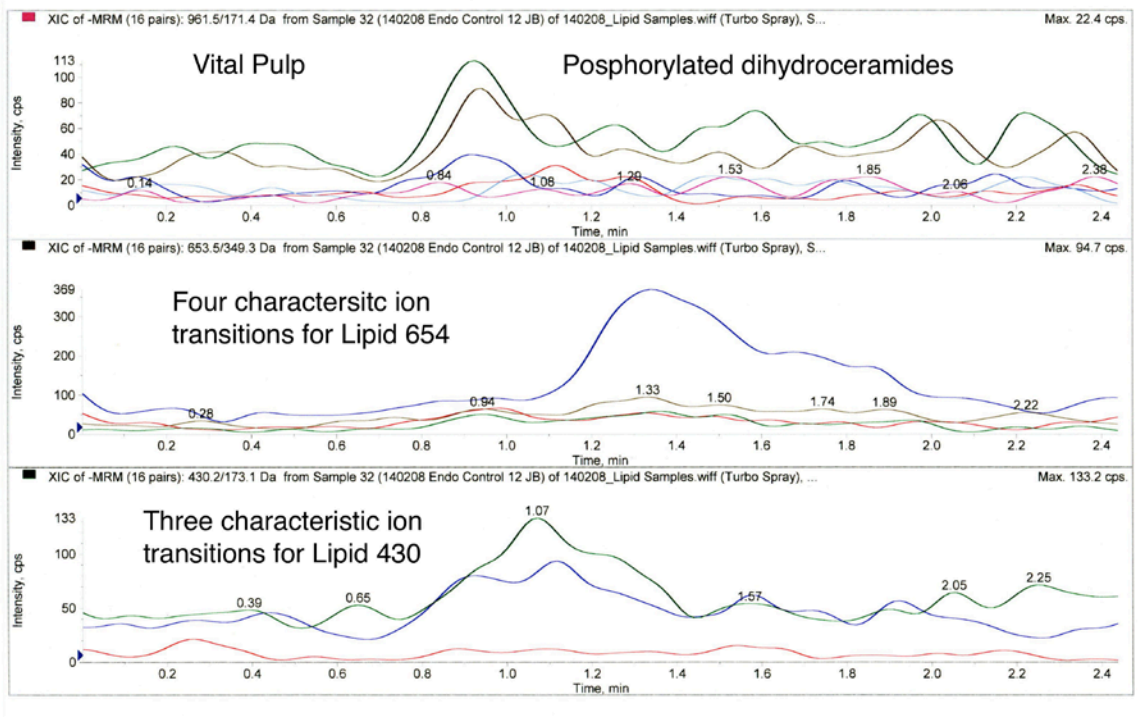


Figure 6: Multiple Reaction Monitoring of samples comparing Lipid 654 amounts in a tooth with a vital pulp. The intensity of Lipid 654, phosphorylated dihydroceramides, and Lipid 430 is minimal in the tooth with a vital pulp.

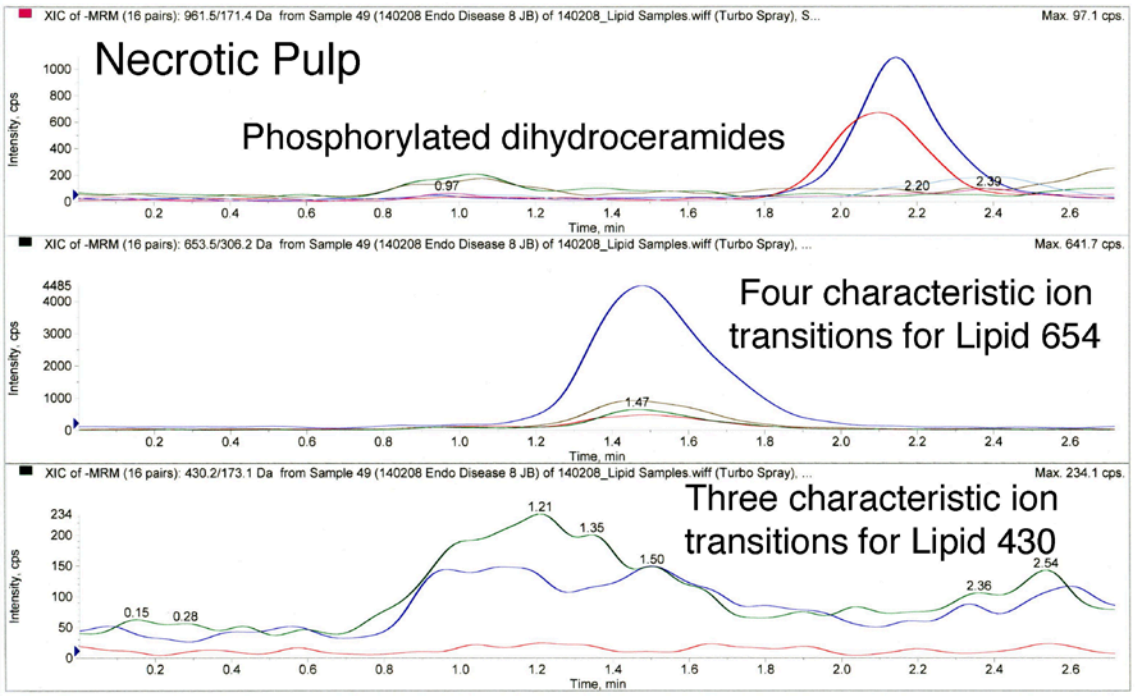


Figure 7: Multiple Reaction Monitoring of samples comparing Lipid 654 amounts in a tooth with a necrotic pulp. The intensity of Lipid 654 is significantly higher than Lipid 654 from vital pulps (see Figure 6) and phosphorylated dihydroceramides and Lipid 430, indicating substantially more Lipid 654 is present in teeth with necrotic pulps.

Comparison of Lipid 654 levels in control teeth with vital pulps and teeth with necrotic pulps using multiple reaction monitoring-mass spectrometry revealed significantly more ($P < .000001$) Lipid 654 was recovered in necrotic teeth (see Figure 8).

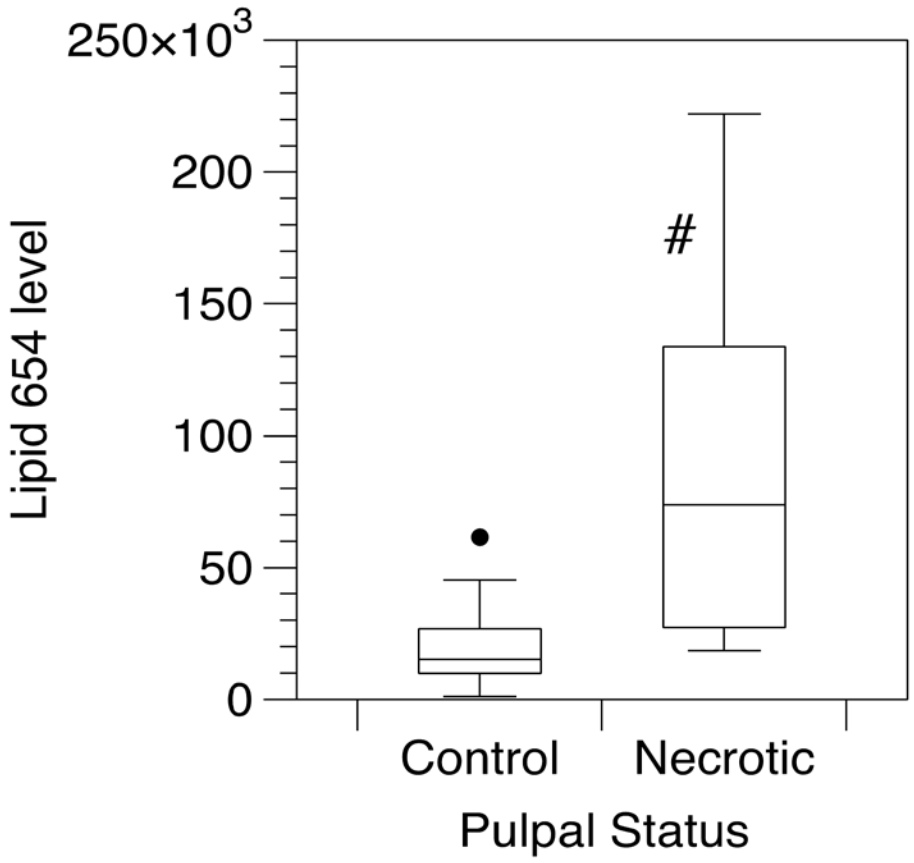


Figure 8: Comparison of Lipid 654 levels in lipid extracts from control teeth with vital pulps and teeth with necrotic pulps using multiple reaction monitoring-mass spectrometry. The dot indicates an outlier in the control samples. Significantly more ($P < .000001$) Lipid 654 was recovered in necrotic pulp lipid extracts (indicated by #).

Outliers were observed in both the teeth with vital pulps and teeth with necrotic pulps, thus a Whisker plot and a Mann-Whitney U test were used to evaluate for significance.

Discussion

Recent studies have shown that *Porphymonas* species are one of the most prevalent bacterial species in teeth with primary and persistent apical periodontitis

(37,38). Specifically, *P. endodontalis* has been identified in numerous studies as a predominant species harbored within an infected root canal space (39, 40, 41). However, little is known as to how this microorganism contributes to periapical bone resorption, as seen in apical periodontitis. Considerable attention has focused on specific bacterial components and/or by-products that are released by microorganisms into their surrounding environments. A previous investigation demonstrated that exposure of human gingival fibroblasts to specific phospholipids of *P. gingivalis* significantly potentiated interleukin-1α (IL-1α)-mediated prostaglandin secretion and promoted marked changes noted in fibroblast morphology suggesting either apoptosis or necrosis (9). *P. endodontalis* is phenotypically close to *P. gingivalis* and both species are expected to produce similar lipids. To date, the specific serine lipid fractions produced by *P. endodontalis* have not been characterized. By using HPLC together with mass spectrometric/nuclear magnetic resonance spectroscopy, we have identified a new serine lipid class of *P. endodontalis*, Lipid 654, that acts as a ligand for human and mouse TLR2.

While lipid 654 shares most structural characteristics with the previously described lipid class that includes Flavolipin (23, 24, 25, 34) and Topostin (42), we found significant differences between lipid 654 and Flavolipin in their respective biological activities. Flavolipin was named after the organisms of the *Flavobacterium* genus, from which it was originally isolated. According to previous reports, Flavolipin represents approximately 20% of the total cellular lipids of *Flavobacterium meningosepticum* and is capable of acting as a TLR4 ligand (23, 25). In contrast, the results of our study demonstrated that Lipid 654 does not activate via TLR4 but instead functions as a ligand

for TLR2. Since both lipid 654 and lipid 430 act as TLR2 ligands but do not appear to activate through engagement of TLR4, it is possible that a lipid contaminant in the Flavolipin preparations accounted for the previously reported TLR4 engagement (23).

An additional important distinction between Flavolipin and lipid 654 is that, while only *Flavobacterium* species were originally reported to produce Flavolipin (23, 24), we now report that lipid 654 is produced by more than *Flavobacterium* species. In addition to *P. gingivalis* and *P. endodontalis*, analysis of lipid extracts from *Prevotella intermedia*, *Tannerella forsythia*, *Capnocytophaga ochracea*, *Capnocytophaga gingivalis*, and *Capnocytophaga sputigena* revealed that each of these oral *Bacteroidetes* produce the lipid 654 class (data not shown). Selected intestinal *Bacteroidetes,* including *Prevotella copri*, *Parabacteroides merdea*, *Parabacteroides distasonis*, *Bacteroides fragilis*, *Bacteroides stercoris*, *Bacteroides uniformis*, and *Bacteroides vulgatis*, also produce the lipid 654 class (data not shown). A complete characterization of commensal organisms capable of producing lipid 654 and lipid 430 will be the focus of future studies. Nevertheless, our evidence thus far indicates that lipid 654 is produced by a wide variety of *Bacteroidetes* species and that, unlike flavolipin, it is not produced exclusively by *Flavobacterium* species (23, 25).

Monocyte Raw 264.7 cells were plated and treated with vehicle control, *P. endodontalis* Lipid 654 extract and *P. gingivalis* Lipid 654. The Lipid 654 fraction of *P. endodontalis* lipids was evaluated in parallel with the total lipid extracts. After 24 hours, a significant increase in tumor necrosis factor-alpha (TNF-α) production via ELISA analysis compared to control was observed for all Lipid fractions. An increase in activity was observed in total lipid and Lipid 654 from *P. gingivalis* compared to Lipid 654 from

P. endodontalis. We suspect that this may be due to the relative amount of Lipid 654 to lipid 430, as the amount lipid 430 may influence the activation the Monocyte Raw 264.7 cells. Further research is needed to evaluate the ability of Lipid 430 and Lipid 654 to activate Monocyte Raw 264.7 cells. The Monocyte Raw 264.7 cells were also pretreated for one hour with 1 μg/ml of mouse anti-TLR2 antibody before treatment with Lipid 654. A significant reduction of activity by both total lipid and Lipid 654 of *P. endodontalis* was observed. We therefore conclude from this evidence that lipid 654 is a structurally unique TLR2 ligand derived from *P. gingivalis* and *P. endodontalis*.

Using HEK293 cells stably transfected with TLR2 along with other relevant receptors, we found that lipid 654 of *P. endodontalis* activates human cells via TLR2. Lipid 654 contains two fatty acids held in an ester linkage and will not dissolve in aqueous solvent unless it is sonicated to form liposome preparations. Previous studies have found that activation of TLR2-expressing HEK293 cells was not mediated by the serine lipid preparations of *P. gingivalis*. With normal-phase HPLC fractionation, Lipid 654 of *P. gingivalis* and *P. endodontalis* elutes slightly earlier than the PE DHC lipids. With the use of state-of-the-art HPLC equipment, which was not available at the time of our earlier work, the Lipid 654 class can now be isolated largely free of PE DHC lipids. Since the purified PE DHC lipids demonstrate no capability to activate human cells through TLR2, we conclude that the previously reported activity of PE DHC lipids of *P. gingivalis* to engage TLR2 in mouse dendritic cells (43) was likely related to contamination of PE DHC lipids with the lipid 654 class.

Since in vitro studies demonstrate that lipid 654 and lipid 430 activate TLR2-expressing HEK cells, Clark et al. tested these lipids on mice to see if the same activation

was seen in vivo (11). Lipid 654 or lipid 430 was injected into either wild type mice (WT) or TLR2-dificient mice (TRL2-/-), and the effects on serum levels of the chemokine CCL2 were measured 4 hours later. CCL2, also known as monocyte chemo-attractant protein 1, plays a major role in mediating the migration of inflammatory macrophages into tissue sites of inflammation. Previous studies have demonstrated that administration of TLR agonists to mice can result in expression of serum CCL2, and this chemokine has been suggested to be important in the pathogenesis of autoimmune diseases. It was observed that administration of either lipid 654 or lipid 430 to WT mice resulted in a significant increase in serum CCL2 levels, however in the TLR2-/- mice, administration of the lipid 654 or lipid 430 resulted in no increase in serum CCL2. These results demonstrate that both lipid 654 and lipid 430 have pro-inflammatory effects in vivo and that these effects are dependent on TLR2

In this study, we evaluated lipid extracts of human impacted third molars using multiple reaction monitoring MS, specifically quantifying the Lipid 654 class. We found these lipid extracts contained minimal amounts of lipid 654 and essentially no lipid 430 (data not shown). These results suggest that in human tissues not directly exposed to bacteria, these lipids are not recovered in appreciable levels. In necrotic pulps, a substantial level of Lipid 654 was recovered, suggesting that this lipid may be an important part of the pulpal and periapical disease process. Worth noting is the amount of Lipid 430 did not increase substantially in the teeth with necrotic pulps. Lipid 654 was observed in levels far exceeding the amounts seen in *P. endodontalis* or *P. gingivalis*, suggesting that this lipid accumulates in the pulp space of teeth with necrotic pulps. Recent studies revealed that lipid extracts from diseased human gingival tissue, carotid

atheroma, and serum demonstrate lipid 654 in levels far exceeding the phosphorylated dihydroceramide lipids of *P. gingivalis* (26). This suggests that Lipid 654 is present in low levels in the blood stream, thus low amounts of Lipid 654 were observed due to the pulpal microvasculature. Further research is needed to examine the production and activity of Lipid 430 in the necrotic pulp system.

Although we have previously shown that LPS and lipid A of *P. gingivalis* are significantly contaminated with the phosphorylated dihydroceramide lipids (22), we have now determined that LPS of *P. gingivalis* prepared according to the methods of Yi and Hacket and Caroff (44, 45) contains an average 3.81% by weight of lipid 654. LPS contamination with lipid 654 supports the conclusion that the TLR2 activity previously attributed to be LPS/lipid A of *P. gingivalis* (46) is due in part to the presence of the 654 lipid class. However, other factors could be involved, as described below. A more recent report (16) indicated that the same phospholipid extraction method used in our laboratory (35, 36) yields an aqueous soluble factor from *P. gingivalis* that engages TLR2/TLR1 to a greater extent than the total lipids recovered in the organic solvent. We recently confirmed that lipid 430 and lipid 654 can be recovered from the aqueous extract of *P. gingivalis* following acidification of the aqueous phase with acetic acid and extraction of lipids into chloroform. The lipid 430 present in the aqueous phase alone could have produced the TLR2 effects observed by Jain et al. (16). Jain et al. also concluded that an aqueous soluble TLR2/ TLR1 agonist copurifies with LPS and is released when the LPS is hydrolyzed to produce lipid A. This LPS-associated factor is attenuated by lipoprotein lipase treatment (16). Lipoprotein lipase will hydrolyze ester-linked fatty acids from triglycerides of lipoproteins. While lipid 430 does not contain an ester-linked fatty acid,

the lipid 654 class does contain an ester-linked fatty acid. Although highly purified lipid 654 is not soluble in aqueous solvents, LPS, which is soluble in aqueous solutions and is contaminated with lipid 654 and lesser amounts of lipid 430 (data not shown), likely acts as a carrier for lipid 654 in aqueous extracts of *P. gingivalis* and *P. endodontalis*. It is also possible that another TLR2 ligand or a lipoprotein that has not yet been identified is present in the aqueous extracts of *P. endodontalis*. However, the previously reported lipopeptide of *P. gingivalis* (47, 48), which is soluble in aqueous solutions, was not the primary TLR2/TLR1 ligand present in the *P. gingivalis* aqueous extract. Until the putative water-soluble factor of *P. gingivalis* described by Jain et al. (16) is isolated and structurally characterized, lipid 430 and lipid 654 constitute the primary TLR2 ligands that are recovered in the aqueous extract of *P. gingivalis*. Future work will focus on the water-soluble TLR2 factors produced by *P. gingivalis*.

Although many virulence factors of *P. gingivalis* have been proposed to contribute to periodontal bone and tissue destruction (16, 28, 29), we have reported that various complex lipids of *P. gingivalis* are readily detected in periodontal disease sites without concurrent bacterial invasion or LPS recovery (30, 31). We have now determined that specific lipid classes that are relatively minor constituents of this organism are responsible for activation of TLR2 and that dihydroceramide lipids are not responsible for these TLR2-mediated biological responses. Our findings are consistent with those of other investigators who have reported that *P. gingivalis* colonization of teeth in experimental animals mediates bone loss through engagement of TLR2 (28, 32, 33). We believe that the lipid 654 and lipid 430 classes produced by *P. gingivalis* and other oral *Bacteroidetes* play a critical role in the development of destructive periodontal

disease by promoting bone loss and inhibition of bone formation, as we have previously reported (34). We further suggest that lipid 654 and lipid 430 may contribute to the development of other chronic inflammatory diseases of humans, where these bacterial lipids accumulate to substantial levels due to contributions from microbes of the oral cavity, gastrointestinal tract, and other anatomical sites.

In summary, this investigation identified a serine lipid class produced by *P. endodontalis* that acts as a newly defined ligand for TLR2. This lipid classes is comprised of a group of lipid species with a serine group, a glycine group, and amide-linked and ester linked fatty acid side chains, and has the same structure and biological activity as the serine lipid previously isolated from *P. gingivalis*. However, it is unclear at this time if the lipid species of lipid 430 represent precursor or breakdown products (or both) for the constituent lipid species of lipid 654, and this will be a focus of future investigations. It is notable that the lipid 654 and lipid 430 classes differ substantially in their solubility characteristics in aqueous solvents. We believe the different solubility characteristics and the enzyme susceptibilities of these lipids could provide a novel framework for different modes of local delivery and innate immune system activation by these lipids. Thus, the two classes of lipids working together could potentially result in the both bone and soft tissue destruction that is associated with chronic periodontitis and apical periodontitis in humans. Further research will clarify these questions.

References Cited:

1. Fabricius L, Dahlen G, Ohman AE, Moller AJ. Predominant indigenous oral bacteria isolated from infected root canals after varied times of closure. Scand J Dent Res 1982;90(2):134-144.
2. Farber PA, Seltzer S. Endodontic microbiology. I. Etiology. J Endod 1988;14(7):363-371.
3. Sakamoto M, Rocas IN, Siqueira JF, Jr., Benno Y. Molecular analysis of bacteria in asymptomatic and symptomatic endodontic infections. Oral Microbiol Immunol 2006;21(2):112-122.
4. Van Winkelhoff AJ, van Steenbergen TJ, de Graaff J. *Porphyromonas (Bacteroides) endodontalis*: its role in endodontal infections. J Endod 1992;18(9):431-434.
5. Hosoya S, Matsushima K. Stimulation of interleukin-1 beta production of human dental pulp cells by *Porphyromonas endodontalis* lipopolysaccharide. J Endod 1997;23(1):39-42.
6. Ko HJ, Lim SS. Production of macrophage inflammatory protein (MIP)-1alpha and MIP-1beta by human polymorphonuclear neutrophils stimulated with *Porphyromonas endodontalis* lipopolysaccharide. J Endod 2002;28(11):754-757.
7. Ogura N, Shibata Y, Kamino Y, Matsuda U, Hayakawa M, Oikawa T, et al. Stimulation of interleukin-6 production of periodontal ligament cells by *Porphyromonas endodontalis* lipopolysaccharide. Biochem Med Metab Biol 1994;53(2):130-136.
8. Tang Y, Sun F, Li X, Zhou Y, Yin S, Zhou X. *Porphyromonas endodontalis* lipopolysaccharides induce RANKL by mouse osteoblast in a way different from that of *Escherichia coli* lipopolysaccharide. J Endod 2011;37(12):1653-1658.
9. Nichols FC, Riep B, Mun J, Morton MD, Bojarski MT, Dewhirst FE, et al. Structures and biological activity of phosphorylated dihydroceramides of *Porphyromonas gingivalis*. J Lipid Res 2004;45(12):2317-2330.
10. Nichols FC, Riep B, Mun J, Morton MD, Kawai T, Dewhirst FE, et al. Structures and biological activities of novel phosphatidylethanolamine lipids of *Porphyromonas gingivalis*. J Lipid Res 2006;47(4):844-853.
11. Clark RB, Cervantes JL, Maciejewski MW, Farrokhi V, Nemati R, Yao X, Anstadt E, Fujiwara M, Wright KT, Riddle C, La Vake CJ, Salazar JC, Finegold S, Nichols FC. Serine Lipids of *Porphyromonas gingivalis* Are Human and Mouse Toll-Like Receptor 2 Ligands. Infect Immun 2013;81(9):3479-3489.
12. De Oliveira JCM, Siqueira JF, Gabriela AB, Hirata Jr. R, Andrade AFB. Detection of *Porphyromonas endodontalis* in Infected Root Canals by 16s rRNA Gene-Directed Polymerase Chain Reaction. Journal of Endodontics 2000;26:729-732
13. Dongyou L. Molecular Detection of Human Bacterial Pathogens. CRC Press 2011:570-571
14. Raetz CR, Whitfield C. Lipopolysaccharide Endotoxins. Annu. Rev. Biochem. 2002;71:635-700.
15. Zhang P, Liu J, Xu Q, Garber G, Feng X, Michalek SM, Katz J. TLR2-dependent Modulation of Osteoclastogenesis by Porphyromonas gingivalis through Differential Induction of NFATc1 and NF-kB. J. Biol. Chem. 2011;286:24159-24169.

16. Jain S, Coats SR, Chang AM, Darveau RP. A Novel Class of Lipoprotein Lipase-Sensitive Molecules Mediates TLR2 Activation by *Prophyromonas gingivalis*. Infect. Immun. 2013;1-47.
17. Burns E, Bachrach G, Shapira L, Nussbaum G. Cutting Edge: TLR2 is Required for the Innate Response to *Porphyromonas gingivalis*: Activation Leads to Bacterial Persistence and TLR2 Deficiency Attenuates Induced Alveolar Bone Resorption. J. Immunol 2006;177:8296-8300.
18. Hirschfeld M, Weis JJ, Toshchakov V, Salkowski CA, Cody MJ, Ward DC, Qureshi N, Michalek SM, Vogel SN. Signaling by Toll-Like Receptor 2 and 4 Agonist Results in Differential Gene Expression in Murine Macrophages. Infection and Immunity 2001;69:1477-1482.
19. Means TK, Wang S, Lien E, Yoshimura A, Golenbock TF, Fenton MJ. Human Toll-Like Receptors Mediate Cellular Activation by *Mycobacterium tuberculosis*. Immunol 1999;163:3920-3927.
20. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS Signaling in C3H/HeJ and C57BL/10ScCr Mice: Mutations in *Tlr4* Gene. Science 1998;282:2085-2088.
21. Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ. Cutting Edge: Repurification of Lipopolysaccharide Eliminates Signaling Through Both Human and Murine Toll-Like Receptor 2. J Immunol 2001;165:618-622.
22. Nichols FC, Bajrami B, Clark RB, Housley W, Yao X. Free Lipid A Isolated from *Prophyromonas gingivalis* Lipopolysaccharide Is Contaminated with Phosphorylated Dihydroceramide Lipids: Recovery in Diseased Dental Samples. Infection and Immunity 2012;80(2):860-874.
23. Gomi K, Kawasaki K, Kawai Y, Shiozaki M, Nishijima M. 2002. Toll-like receptor 4-MD-2 complex mediates the signal transduction induced by flavolipin, an amino acid-containing lipid unique to *Flavobacterium meningosepticum*. J. Immunol. 168:2939 –2943.
24. Kawai Y, Yano I, Kaneda K. 1988. Various kinds of lipoamino acids including a novel serine-containing lipid in an opportunistic pathogen *Flavobacterium*. Their structures and biological activities on erythrocytes. Eur. J. Biochem. 171:73– 80.
25. Kawasaki K, Gomi K, Kawai Y, Shiozaki M, Nishijima M. 2003. Molecular basis for lipopolysaccharide mimetic action of Taxol and flavolipin. J. Endotoxin Res. 9:301–307.
26. Nichols FC, Yao X, Bajrami B, Downes J, Finegold SM, Knee E, Gallagher JJ, Housley WJ, Clark RB. 2011. Phosphorylated dihydroceramides from common human bacteria are recovered in human tissues. PLoS One 6(2):e16771. doi:10.1371/journal.pone.0016771.
27. Ukai T, Yumoto H, Gibson FC, III, Genco CA. 2008. Macrophage- elicited osteoclastogenesis in response to bacterial stimulation requires Toll-like receptor 2-dependent tumor necrosis factor-alpha production. Infect. Immun. 76:812– 819.
28. PapadopoulosG,WeinbergEO,MassariP,GibsonFC,III,WetzlerLM, Morgan EF, Genco CA. 2013. Macrophage-specific TLR2 signaling mediates pathogen-induced TNF-dependent inflammatory oral bone loss. J. Immunol. 190:1148 –1157.
29. Gibson FC, III, Hong C, Chou HH, Yumoto H, Chen J, Lien E, Wong J, Genco CA.

Innate immune recognition of invasive bacteria accelerates atherosclerosis in apolipoprotein E-deficient mice. Circulation 2004;109:2801–2806.
30. Nichols FC. Distribution of 3-hydroxy-C17:0 in subgingival plaque and gingival tissue samples: relationship to adult periodontitis. Infect. Immun. 1994;62:3753–3760.
31. Nichols FC, Rojanasomsith K. *Porphyromonas gingivalis* lipids and diseased dental tissues. Oral Microbiol. Immunol. 2006;21:84 –92.
32. Gibson FC, III, Ukai T, Genco CA. Engagement of specific innate immune signaling pathways during *Porphyromonas gingivalis* induced chronic inflammation and atherosclerosis. Front. Biosci. 2008;13:2041–2059.
33. Liang S, Krauss JL, Domon H, McIntosh ML, Hosur KB, Qu H, Li F, Tzekou A, Lambris JD, Hajishengallis G. The C5a receptor impairs IL-12-dependent clearance of *Porphyromonas gingivalis* and is required for induction of periodontal bone loss. J. Immunol. 2011;186:869 – 877.
34. Wang YH, Jiang J, Zhu Q, Alanezi AZ, Clark RB, Jiang X, Rowe DW, Nichols FC. *Porphyromonas gingivalis* lipids inhibit osteoblastic differentiation and function. Infect. Immun. 2010;78:3726 –3735.
35. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911-917.
36. Garbus J, DeLuca HF, Loomas ME, Strong FM. Rapid incorporation of phosphate into mitochondrial lipids. J Biol Chem 1968;238:59-63.
37. Sassone LM, Fidel RA, Faveri M, Figueiredo L, Fidel SR, Feres M. A microbiological profile of unexposed and exposed pulp space of primary endodontic infections by checkerboard DNA DNA hybridization. J Endod 2012;38(7):889-893.
38. Rocas IN, Siqueira JF, Debelian GJ. Analysis of symptomatic and asymptomatic primary root canal infections in adult Norwegian patients. J Endod 2011;37(9):1206-1212.
39. Van Winkelhoff AJ, Carlee AW, de Graaff J. *Bacteroides endodontalis* and other black-pigmented Bacteroides species in odontogenic abscesses. Infect Immun 1985;49:494-497.
40. Sundqvist G, Eckerbom MI, Larrson AP, Sjogren UT. Capacity of anaerobic bacteria from necrotic dental pulps to induce purulent infections. Infect. Immun. 1979;25(2):685-693.
41. Siqueira JF, Rocas IN, De Uzeda M, Colombo AP, Santos KR. Comparison of 16S rDNA-based PCR and checkerboard DNA-DNA hybridization for detection of selected endodontic pathogens. J Med Microbiol. 2002;51(12):1090-1096.
42. Shiori T, Terao Y, Irako N, Aoyama T. Synthesis of topostins B567 and D654 (WB-3559D, flavolipin), DNA topoisomerase I inhibitors of bacterial origins. Tethrahedron 1998;54:15701-15710.
43. Nichols FC, Housley WJ, O'Conor CA, Manning T, Wu S, Clark RB. Unique lipids from a common human bacterium represent a new class of toll-like receptor 2 ligands capable of enhancing autoimmunity. Am. J. Pathol. 2009;175:2430-2438.
44. Yi EC, Hackett M. Rapid isolation method for lipopolysaccharide and lipid A from Gram-negative bacteria. Analyst 2000;125:651-656.

45. Cann-Moisan C, Caroff J, Girin E. Determination of flavin adenine dinucleotide in biological tissues by high-performance liquid chromatography with electrochemical detection. J. Chromatogr. 1988;442:441-443.
46. Darveau RP, Pham TT, Lemley K, Reife RA, Bainbridge BW, Coats SR, Howald WN, Way SS, Hajjar AM. *Porphyromonas gingivalis* lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. Infect. Immun. 2004;72:5041-5051
47. Hashimoto M, Asai Y, Ogawa T. Separation and structural analysis of lipoprotein in a lipopolysaccharide preparation from *Porphyromonas gingivalis*. Int. Immunol. 2004;16:1431-1437.
48. Makimura Y, Asai Y, Taiji Y, Sugiyama A, Tamai R, Ogawa T. Correlation between chemical structure and biological activities for *Porphyromonas gingivalis* synthetic lipopeptide derivatives. Clin. Exp. Immunol. 2006;146:159-168.